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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Casida <i>et al.</i>	Confirmation No.:	3630
Application No.:	10/776,767	Art Unit:	1651
Filed:	February 10, 2004	Examiner:	Afremova, Vera
For:	NON-OBLIGATE PREDATORY BACTERIUM <i>BURKHOLDERIA</i> <i>CASIDAE</i> AND USES THEREOF		
		Attorney Docket No.:	8014-020-999

**DECLARATION OF JOSEPH O. FALKINHAM, III, PH.D.
UNDER 37 C.F.R. § 1.132**

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, JOSEPH O. FALKINHAM, III, Ph.D., do declare as follows:

1. I am currently Professor of Microbiology, Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. I am a co-inventor of the above-identified patent application, No. 10/776,767 (the “767 application”), which has been assigned to Virginia Tech Intellectual Properties, Inc. It is my understanding that intellectual property made by employees of Virginia Polytechnic Institute and State University is generally assigned to Virginia Tech Intellectual Properties, Inc.

2. Further, during the four years that I served in the U.S. Air Force Biomedical Sciences Corps, I performed *in vitro* antibiotic susceptibility measurements to guide the choice of antibiotics and dosage by physicians treating patients. My education and experience are summarized on my Curriculum Vitae, which is attached hereto as Exhibit 1.

3. It is my understanding that there is an outstanding Office Action, mailed February 5, 2007, in the ‘767 application. I have reviewed the Office Action and have been asked to evaluate first whether a person skilled in the art at the time the ‘767 application was first filed (*i.e.*, April 23, 1997), based on the teaching of the specification of the ‘767 application coupled with knowledge common in the art at the time the ‘767 application was

first filed, would have concluded that the inventors had possession of an effective dose of an antimicrobial preparation comprising a substantially pure culture or suspension of *Burkholderia casidae* or variant thereof, a cell-free filtrate or cell fraction prepared from a substantially pure culture or suspension of *Burkholderia casidae* or variant thereof, or a cell-free filtrate or cell fraction prepared from an inactivated substantially pure culture or suspension of *Burkholderia casidae* or variant thereof, for treating or inhibiting in an animal a disease caused by a microorganism (see ¶¶4-7 below). I also have been asked to evaluate whether a person skilled in the art at the time the '767 application was first filed, based on the teaching of the specification of the '767 application coupled with knowledge common in the art at the time the '767 application was first filed, and using only routine experimentation, would have been able to determine an effective dose of an antimicrobial preparation comprising a substantially pure culture or suspension of an appropriate *Burkholderia casidae* or variant thereof, a cell-free filtrate or cell fraction prepared from a substantially pure culture or suspension of *Burkholderia casidae* or variant thereof, or a cell-free filtrate or cell fraction prepared from an inactivated substantially pure culture or suspension of *Burkholderia casidae* or variant thereof, for treating or inhibiting in an animal a disease caused by a microorganism (see ¶¶8-15 below). Third, I have been asked to evaluate whether a person skilled in the art at the time the '767 application was first filed would have recognized that certain microorganisms that could be isolated from soil are also found in the normal flora of animals or humans (see ¶¶16-17 below).

4. The specification of the '767 application as filed describes the term "effective dose" and methods for assessing such dose useful for treating or inhibiting in an animal a disease caused by a microorganism (see, e.g., paragraph bridging pages 30 and 31). For example, the specification describes a disc diffusion test useful for measuring the inhibitory activity of *Burkholderia casidae* strain 2.2N culture, fraction and/or cells on various animal pathogens, such as *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus niger*, *Staphylococcus aureus*, and *Mycobacterium smegmatis*, in which the level of inhibition was assessed by measuring the diameter of the zones of inhibition of the pathogen growth surrounding the spots of *Burkholderia casidae* strain 2.2N culture, fraction and/or cells (see, e.g., Section 6.2.4 on page 53; Tables 8 and 9 at page 54; Table 10 at page 55; Table 17 at page 65; Tables 19 and 20 at page 66; and Table 21 at page 68).

5. The disc diffusion test described and utilized in the '767 application is the most widely used *in vitro* susceptibility testing method for the selection and dosing of an antimicrobial agent, and has been accepted by the Food and Drug Administration (FDA) and as a standard by the National Committee on Clinical Laboratory Standards (NCCLS). See, e.g., Sherris, J. C. & Washington, II, J.A., "Section V. Laboratory Tests in Chemotherapy, Chapter 41, General Considerations," in Manual of Clinical Microbiology (3d ed. 1980), American Society for Microbiology, Washington, D.C., E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant eds., page 447, col. 1, ¶2 (hereinafter "Sherris," a copy of which is attached as Exhibit 2); and W. Robert Bailey & Elvyn G. Scott, Diagnostic Microbiology (4th ed. 1974), C. V. Mosby Company, St. Louis, MO, Chapter 34, "Determination of susceptibility of bacteria to antimicrobial agents," page 318, col. 2, ¶2 (hereinafter "Bailey," a copy of which is attached as Exhibit 3). It would have been clear to a person skilled in the art at the time the '767 application was first filed that standard tests such as the disc diffusion tests described in the specification of the '767 application could be used to routinely calculate the dose of *Burkholderia casidae* (or variant thereof), suspension, cell-free filtrate, and/or cell fraction that would be effective (*i.e.*, "effective dose") to treat or inhibit in an animal a disease caused by a microorganism. For example, based on the diameter of the zones of inhibition, such person could routinely calculate the lowest concentration of *Burkholderia casidae* strain 2.2N culture, fraction and/or cells necessary to either inhibit (that is, minimal inhibitory concentration, MIC) or kill (that is, minimal bactericidal concentration, MBC) the pathogen, and therefore, determine the effective dose of *Burkholderia casidae* strain 2.2N culture, fraction and/or cells useful for treating or inhibiting in an animal a disease caused by the pathogen. See Bailey, sections entitled "Reading of results" and "Interpretation of zone sizes" on pages 321-324.

6. It would have also been clear to a person skilled in the art at the time the '767 application was first filed that *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus niger*, *Staphylococcus aureus*, and *Mycobacterium smegmatis* were known to cause disease in animals (see, e.g., Schmid *et al.*, "Evidence for nosocomial transmission of *Candida albicans* obtained by Ca3 fingerprinting," J Clin Microbiol. 1995 May;33(5):1223-30, page 1223, col. 1, ¶1; Sullivan *et al.*, "Persistence, replacement, and microevolution of *Cryptococcus neoformans* strains in recurrent meningitis in AIDS patients," J Clin Microbiol. 1996 Jul;34(7):1739-44, page 1739, col. 1, ¶1; Luce *et al.*, "Invasive aspergillosis presenting as pericarditis and cardiac tamponade," Chest. 1979 Dec;76(6):703-5, page 703, col. 2, ¶2;

Blouse *et al.*, "Colonization and infection of newborn infants caused by bacteriophage-group II *Staphylococcus aureus* strains," J Clin Microbiol. 1979 Oct;10(4):604-6, page 604, col. 1, ¶1; and Newton *et al.*, "Soft-tissue infection due to *Mycobacterium smegmatis*: report of two cases," Clin Infect Dis. 1993 Apr;16(4):531-3, page 531, col. 1, ¶1, copies of which are attached as Exhibits 4-8, respectively). In addition, it would have been clear to a person skilled in the art at the time the '767 application was first filed that certain other members of *Candida* (e.g., *Candida glabrata*), *Cryptococcus* (e.g., *Cryptococcus laurentii*), *Aspergillus* (e.g., *Aspergillus fumigatus* and *Aspergillus flavus*), *Staphylococcus* (e.g., *Staphylococcus lugdunensis*, *Staphylococcus intermedius* and *Staphylococcus saprophyticus*), and *Mycobacterium* (e.g., *Mycobacterium tuberculosis*) are animal pathogens, based on case studies and models available to a person skilled in the art at the time the '767 application was first filed.

7. Therefore, in my judgment and opinion, a person skilled in the art at the time the '767 application was first filed, based on the teaching of the specification of the '767 application coupled with knowledge common in the art at the time the '767 application was first filed, would have concluded that the inventors had possession of an effective dose of an antimicrobial preparation comprising a substantially pure culture or suspension of *Burkholderia casidae* or variant thereof, a cell-free filtrate or cell fraction prepared from a substantially pure culture or suspension of *Burkholderia casidae* or variant thereof, or a cell-free filtrate or cell fraction prepared from an inactivated substantially pure culture or suspension of *Burkholderia casidae* or variant thereof, for treating or inhibiting in an animal a disease caused by a microorganism.

8. As explained in Paragraphs 9-11 below, a person skilled in the art at the time the '767 application was first filed, based on the teaching of the specification of the '767 application coupled with knowledge common in the art at the time the '767 application was first filed, and using only routine experimentation, would have been able to identify a non-pathogenic *Burkholderia casidae* or variant thereof useful for treating or inhibiting in an animal a disease caused by a microorganism.

9. First, the specification of the '767 application as filed describes several methods for isolating and identifying a *Burkholderia casidae* or variant thereof, including strain 2.2 N, useful to treat or inhibit in an animal a disease caused by a microorganism (see,

e.g., Section 6.1 on pages 32-49). As part of such isolation and identification, it is possible that the skilled artisan would desire to test for possible pathogenicity of the isolated *Burkholderia casidae* or variant. At the time the '767 application was first filed, the skilled artisan would have been able to routinely conduct such testing. For example, by the time the '767 application was first filed, one of skill in the art would have been aware that pathogenic strains of the *Burkholderia* genus exhibit a number of virulence genes, where presence or absence in the identified strain could routinely be assessed, which would, thereby, assess the likely pathogenicity of the identified strain.

10. The *Burkholderia casidae* strain 2.2N described in the specification of the '767 application does not display any of the aforementioned virulence genes and is not pathogenic. Moreover, at the time the '767 application was first filed, and even today, no pathogenic *Burkholderia casidae* strain has been identified.

11. With respect to testing for such pathogenicity, it would have been clear to a person skilled in the art at the time the '767 application was first filed that the pathogenicity (if any) of a particular *Burkholderia casidae* isolate could be assessed by routinely testing for particular virulence genes. For example, at the time the '767 application was first filed, it was known that *Burkholderia cepacia*, which has also been known as *Pseudomonas cepacia*, expresses virulence factors such as protease, lecithinase, gelatinase, lipase, and hemolysin. See, e.g., Nakazawa *et al.*, "Characterization of hemolysin in extracellular products of *Pseudomonas cepacia*," J Clin Microbiol. 1987 Feb;25(2):195-8, page 197, col. 1, ¶¶4-5; and Nelson *et al.*, "Virulence factors of *Burkholderia cepacia*," FEMS Immunol Med Microbiol. 1994 Feb;8(2):89-97, page 89, col. 1, ¶1, lines 1-3, and page 92, col. 1, ¶2, lines 1-5, copies of which are attached as Exhibits 9 and 10, respectively. At the time the '767 application was first filed, it was also known that *Burkholderia cepacia* isolates which are pathogenic in patients with cystic fibrosis display a unique set of genetic traits (e.g., *cblA* cable pilus subunit gene, enzyme electrophoretic type 12 (ET12) genotype, IS402-IS1356 hybrid element, mesh (Msh) pili, and *Burkholderia cepacia* epidemic strain (BCESM) marker) that distinguished them from other *Burkholderia casidae* isolates which are recovered from nosocomial and environmental sources. See, e.g., Sun *et al.*, "The emergence of a highly transmissible lineage of *cblI*⁺ of *Pseudomonas (Burkholderia) cepacia* causing CF centre epidemics in North America and Britain," Nat Med. 1995 July;7(1):661-6, page 664, col. 1, ¶2, lines 18-24; Tyler *et al.*, "Identification of IS1356, a new insertion sequence, and its

association with IS402 in epidemic strains of *Burkholderia cepacia* infecting cystic fibrosis patients,” J Clin Microbiol. 1996 Jul;34(7):1610-6, page 1614, col. 1, ¶1, lines 4-8; and Mahenthalingam *et al.*, “Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis,” J Clin Microbiol. 1997 Apr;35(4):808-16, J Bacteriol. 1995 Feb;177(4):1039-52, page 815, col. 2, ¶2, last sentence, copies of which are attached as Exhibits 11-13, respectively).

12. As explained in Paragraphs 13-15 below, a person skilled in the art at the time the ‘767 application was first filed, based on the teaching of the specification of the ‘767 application coupled with knowledge common in the art at the time the ‘767 application was first filed, and using only routine experimentation, would have been able to determine an effective dose of an antimicrobial preparation comprising *Burkholderia casidae* (or variant thereof), suspension, cell-free filtrate, and/or cell fraction for treating or inhibiting in an animal a disease caused by a microorganism.

13. As discussed above in Paragraph 4, the specification of the ‘767 describes the use of a disc diffusion test to measure the effective dose of *Burkholderia casidae* strain 2.2N culture, fraction and/or cells on various animal pathogens. As discussed above in Paragraph 5, a person skilled in the art, based on the knowledge common in the art at the time the ‘767 application was first filed, could use the diameter of the zone of inhibition obtained from the disc diffusion test described in the specification of the ‘767 application to routinely calculate the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the antimicrobial agent, and therefore, routinely calculate the effective dose of the antimicrobial agent.

14. It would have also been clear to such person that other standard tests well known to a skilled artisan at the time the ‘767 application was first filed, such as the dilution test, could routinely be used to calculate an effective dose of *Burkholderia casidae* (or variant thereof), suspension, cell-free filtrate and/or cell fraction for treating or inhibiting in an animal a disease caused by a pathogen. See, *e.g.*, Sherris in Exhibit 2, p. 447, col. 1, last paragraph, to p. 448, col. 1, ¶2; and Bailey in Exhibit 3, p. 317, col. 2, line 1, to p. 318, col. 2, line 3. Like the diffusion test, the dilution test is also a well established *in vitro* susceptibility testing method commonly used by microbiologists and clinicians for the selection and *in vivo*

dosing of an antimicrobial agent. See, e.g., Sherris in Exhibit 2, page 447, col. 1, ¶4; and Bailey in Exhibit 3, page 313, paragraph bridging columns 1 and 2. *In vitro* data is one of many factors necessarily considered during the selection and *in vivo* dosing of an antimicrobial agent. See, e.g., Sherris in Exhibit 2, p. 446, col. 1, ¶¶1-2.

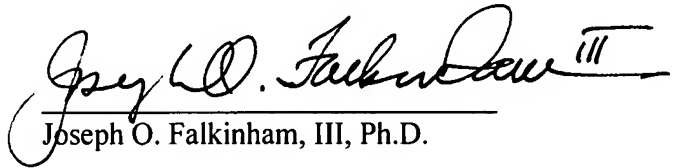
15. Therefore, in my judgment and opinion, a person skilled in the art at the time the '767 application was first filed, based on the teaching of the specification of the '767 application coupled with knowledge common in the art at the time the '767 application was first filed, and using only routine experimentation, would have been able to determine an effective dose of an antimicrobial preparation comprising a substantially pure culture or suspension of an appropriate *Burkholderia casidae* or variant thereof, a cell-free filtrate or cell fraction prepared from a substantially pure culture or suspension of *Burkholderia casidae* or variant thereof, or a cell-free filtrate or cell fraction prepared from an inactivated substantially pure culture or suspension of *Burkholderia casidae* or variant thereof, for treating or inhibiting in an animal a disease caused by a microorganism.

16. As explained below, a person skilled in the art at the time the '767 application was first filed would have recognized that certain microorganisms that could be isolated from soil are also found in the normal flora of animals or humans. For example, *Lactobacilli* can be isolated from soil, corn foliage, and corn silage and are also members of the normal human gut and/or vaginal flora. With respect to members of the *Burkholderia* genus, such as *Burkholderia cepacia* (also known as *Pseudomonas cepacia*), have been recovered from the sputum of humans who lack any evidence of overt infection or disease, and also have been recovered from plants, soils, and water in the natural environment (see, e.g., Govan *et al.*, "*Burkholderia cepacia*: medical, taxonomic and ecological issues," J Med Microbiol. 1996 Dec;45(6):395-407, p. 396, col. 1, ¶3, lines 1-2, and col. 2, ¶3, lines 9-13; Goldman *et al.*, "*Pseudomonas cepacia*: biology, mechanisms of virulence, epidemiology," J Pediatr. 1986 May;108(5 Pt 2):806-12, p. 806, col. 1, ¶1, lines 4-5; p. 806, col. 2, ¶1, lines 12-14; p. 807, col. 1, ¶4, lines 1-4; p. 808, col. 1, ¶2, lines 2-3; and Holmes B., "The identification of *Pseudomonas cepacia* and its occurrence in clinical material," J Appl Bacteriol. 1986 Oct;61(4):299-314, p.305, col. 1, ¶2, lines 1-2; and p. 306, col. 2, ¶3, lines 1-4, copies of which are attached as Exhibits 14-16, respectively).

17. Therefore, in my judgment and opinion, a person skilled in the art at the time the '767 application was first filed, would have recognized that certain microorganisms that could be isolated from soil are also found in the normal flora of animals or humans.

18. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that I make these statements with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the '767 application, and any patent issuing thereon.

Date: June 14, 2007



Joseph O. Falkinham, III, Ph.D.



CURRICULUM VITA

NAME: Joseph Oliver Falkinham, III

BIRTH DATE AND PLACE: May 3, 1942 - Oakland, California

EDUCATION:

- 1970 Intern in Clinical Laboratory Medicine, David Grant USAF
 Medical Center, Travis AFB, California
- 1969 Ph.D., Microbiology, University of California, Berkeley, California
- 1964 A.B., Bacteriology, University of California, Berkeley, California

PROFESSIONAL EMPLOYMENT:

- 1994-present Professor of Microbiology
 Department of Biology
 Virginia Polytechnic Institute and State University
 Blacksburg, VA 24061
- 1980-1993 Associate Professor of Microbiology (tenured)
 Department of Biology
 Virginia Polytechnic Institute and State University
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- 1974-1980 Assistant Professor of Microbiology
 Department of Biology
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- 1972-1974 Fellow in Microbiology and Research Associate
 Department of Microbiology
 University of Alabama Medical Center
 Birmingham, Alabama 35294
 Laboratory of Dr. Roy Curtiss, III
- 1971-1972 Captain USAF (BSC)
 Chief, Laboratory Services
 USAF Hospital
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- 1969-1971 Captain USAF (BSC)
 Chief, Chemistry and Special Chemistry Section
 Department of Pathology
 David Grant USAF Medical Center
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- 1965-1969 Predoctoral Trainee, USPHS and Graduate Teaching Assistant
 Department of Bacteriology, University of California
 Berkeley, California 94720
 Laboratory of Dr. Alvin J. Clark
- 1964-1965 Laboratory Technician and Teaching Assistant
 Department of Soils and Plant Nutrition
 University of California
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 Laboratory of Dr. A. Douglas MacLaren

HONORS

- 2006 Outstanding Teaching Award
 Department of Biological Sciences
 Virginia Polytechnic Institute and State University
 Blacksburg, Virginia
- 2003 Gardner Middlebrook Award for Mycobacteriology
 Sponsored by Becton Dickinson Diagnostic Systems
 American Society for Microbiology Annual Meeting
 Washington, D.C.

INVITED LECTURES

- 2003 Symposium Speaker
 Joint New Zealand and Australia Microbiology Societies
 Auckland, New Zealand
- 2002 Burroughs Wellcome – American Society for Microbiology
 Distinguished Visiting Professor in Microbiology
 University of Texas, El Paso
- Symposium Speaker
 European Society for Mycobacteriology
 Dubrovnik, Croatia
- 2001 Workshop Speaker, Academy of Finland
 Microbes and Man: Health, Nutrition and Environment
 Helsinki, Finland
- 1999 Symposium Speaker, European Congress of Clinical
 Microbiology and Infectious Diseases
 Berlin, Germany
- 1998 Symposium Speaker
 American Thoracic Society/American Lung Association
 Chicago, IL

- 1997 Symposium Speaker, Australian Society for Microbiology
Adelaide, Australia
- 1993 Invited Lecturer, Society for General Microbiology
Exeter, United Kingdom.
- 1986 Divisional Lecturer, American Society of Microbiology,
Washington, D.C.

CONSULTANT

- 1993 - present Chief Scientific Advisor
Dominion Biosciences, Inc., Blacksburg, VA
- 1976 – 1995 Scientific Consultant,
Sybron Chemicals, Inc., Salem, VA
- 1979 - 1993 Lecturer in Biotechnology,
Patent Resources Group, Inc., Washington, D.C.

ADDITIONAL PROFESSIONAL ACTIVITIES

- 1986 Lecturer in Genetic Engineering, U.S. Patent and Trademark Office,
U.S. Department of Commerce, Washington, D. C.
- 1986 Lecturer in Biotechnology, Office of Technology Assessment,
U. S. Congress, Washington, D. C.
- 1982 Visiting Scholar and Lecturer, Pennsylvania State University, Pittsburgh,
Pennsylvania
- 1978 Invited Scientist and Lecturer, Polish Academy of Sciences and
University of Warsaw, Warsaw, Poland.
- 1975 Visiting Scientist, Department of Microbiology,
University of Virginia Medical School, Charlottesville.

PATENTS

U.S. Patent Number 5,527,677

Water Sample Viral Contamination Detection System

M. Ijzerman, C. Hagedorn, III, and J.O. Falkinham, III

Issued: 18 June 1996

U.S. Patent Number 6,319,497

Non-Obligate Predatory Bacterium *Burkholderia casidae*

J.O. Falkinham, III, C.C. Cain, and E.J. Casida

Issued: 20 November 2001

U.S. Patent Number 6,689,357

Non-Obligate Predatory Bacterium *Burkholderia casidae*

J.O. Falkinham, III, C.C. Cain, and E.J. Casida

Issued: 10 February 2004

PUBLICATIONS

Books

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Editor

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- Falkinham, J. O., III. 2007. Growth in catheter biofilms and antibiotic resistance of *Mycobacterium avium*. *J. Med. Microbiol.* 56:250-254.
- Williams, A. A., E. W. Sugandhi, R. V. Macri, J. O. Falkinham, III, and R. D. Gandour. 2007. Antimicrobial activity of long-chain, water-soluble, dendritic tricarboxylate amphiphiles. *J. Antimicrob. Chemother.* 59:451-458.
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- Culp, C. E., J. O. Falkinham, III, and L. K. Belden. 2007. Identification of the natural bacterial microflora on the skin of eastern newts, bullfrog tadpoles and redback salamanders. *Herpetologica* 63:66-71.
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Synthesis and antimicrobial evaluation of water-soluble, dendritic derivatives of epimeric 5 α -cholestan-3-amines and 5 α -cholestan-3-yl aminoethanoates.
Steroids.

RESEARCH GRANTS AND FINANCIAL SUPPORT

- 2007-2008 Development of a Rapid Assay for Coliphage
United States Environmental Protection Agency (EPA)
\$ 50,000
- 2006-2007 Inhibitory Effects of Selected Chemicals on Mycobacteria
United States Council for Automotive Research
\$ 60,000
- 2003-2005 Studies of Flora and Predator Bacteria of Jordan
National Institutes of Health, Fogarty International Center
International Cooperative Biodiversity Groups
\$ 465,019
- 2004-2005 Adaptation of Rapid Coliphage Detection Assay for Field Use
Environmental Protection Agency, Region 6, Dallas, TX
\$ 31,960
- 2001-2003 Genetic Characterization of Industrial Microorganisms
Virginia Center for Innovative Technology and
American Biosystems, Inc., \$ 45,000
- 1999-2010 Physiological Ecology of Mycobacteria
Applied Microbiology and Genetics, \$ 250,000
- 1998-2005 Genetic Studies of *Burkholderia casidae* strain 2.2 N
Dominion BioSciences, Inc., \$ 101,000
- 1997-1999 PCR Detection of Mycobacteriosis in Aquaculture of Fish
NOAA, Sea Grant Program, \$ 120,000.
- 1996-1999 Occurrence and Control of *Mycobacterium avium* Complex,
American Water Works Association Research Foundation, \$
250,000.
- 1996-1998 Predator Fungicide Development,
Dominion Biosciences, Inc., \$ 175,000.
- 1993-1995 Commercial Development of a Rapid Coliphage Assay,
Virginia Center for Innovative Technology, \$ 128,515.
- 1991-1994 Disseminated *Mycobacterium avium* in AIDS,
National Institute of Allergy and Infectious Disease, \$
267,550.
- 1991-1994 Clofazimine Action in Mycobacteria,
Heiser Program for Research in Leprosy, \$ 18,315.
- 1989-1990 Mechanism of Clofazimine Resistance in *Mycobacterium avium*,

The Potts Foundation, \$ 5,300.

- 1987-1990 Epidemiology of Infection by Atypical Mycobacteria, National Institute of Allergy and Infectious Diseases, \$ 338,208.
- 1986-1987 Comparison of Mitochondrial DNA from Dairy and Beef Cattle Breeds, Biomedical Research Support Grant, \$ 2,100.
- 1983-1986 Epidemiology of Infection by Atypical Mycobacteria, National Institute of Allergy and Infectious Disease, \$ 503,410.
- 1983-1984 Proof of Relatedness of Mercury- and Cadmium-resistant Human and Environmental Isolates of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. Biomedical Research Support Grant, \$ 2,000.
- 1982-1983 Improvement in Silage Fermentation Through Genetic Engineering of Lactobacilli, George A. Jeffreys and Co., Inc. \$ 1,800.
- 1980-1982 Analysis of the *lon*-locus of *Escherichia coli* K-12, National Institute of Allergy and Infectious Disease, \$ 28,733.
- 1979-1982 Epidemiology of Infection by Atypical Mycobacteria, National Institute of Allergy and Infectious Diseases, \$ 350,098.
- 1977-1980 Membrane Involvement in Bacterial Conjugation, National Institute of Allergy and Infectious Diseases, \$ 51,101.
- 1977-1979 Epidemiology of Infection by Atypical Mycobacteria, National Institute of Allergy and Infectious Diseases, \$ 120,323.
- 1977-1978 Occurrence, Distribution, and Detection of Water-borne Bacterial Pathogens, Virginia Office of Water Resources Research, \$ 15,907.

THESES AND DISSERTATIONS DIRECTED

- Butala, N. (MS, Microbiology, 2006), "Nitrate and Nitrite-Reductase Activities in *Mycobacterium avium* A5."
- Steed, K. A. (MS, Microbiology, 2003), "Effect of Biofilm Growth on Antibiotic- and Chlorine-Susceptibility of *Mycobacterium avium* and *Mycobacterium intracellulare*."
- Jewell, S.N. (M.S. Microbiology, 2000), "Purification and Characteristics of a Novel Protease from *Burkholderia ambifaria*, Strain 2.2 N.
- Taylor, R.T. (M.S., Microbiology, 1998), "Chlorine, Chloramine, Chlorine Dioxide,

and Ozone susceptibility of *Mycobacterium avium*."

- Cowen, H. E. (M.S. Microbiology, 1998), "Rapid, Quantitative Assessment of *Mycobacterium avium* Susceptibility to Chlorine Based on the Firefly Luciferase Reporter Gene."
- Jensen, D. M. (Ph.D., Microbiology, 1997), "Genetic Basis for Macrolide Resistance in *Mycobacterium avium*".
- Stanek, J.E. (M.S., Microbiology, 1997), "Development of a Rapid Coliphage Detection Assay".
- Eaton, T. (M.S., Microbiology, 1993), "Epidemiology of *Mycobacterium avium* complex infecting AIDS patients".
- Via, L. E. (Ph.D., Microbiology, 1993), "Insertion sequence IS1141: Discovery, characterization and association with *Mycobacterium avium* colonial variation".
- Jucker, M. T. (Ph.D., Microbiology, 1991), "Identification and characteristics of plasmid homology groups of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*".
- Carlisle, G.C. (M.S. Microbiology, 1991), "Isolation and characterization of the major cytoplasmic membrane protein of *Mycobacterium avium*".
- Warek, U. (M.S., Microbiology, 1990), "Mechanism of action of clofazimine in *Mycobacterium avium* and *Mycobacterium intracellulare*".
- Stormer, R. S. (M.S. Microbiology, 1989), "Differences in antimicrobial susceptibility of pigmented and unpigmented colonial variants of *Mycobacterium avium*".
- Pethel, M. L. (M.S. Microbiology, 1988), "Plasmid-influenced changes in *Mycobacterium avium* catalase".
- Erardi, F.X. (M.S. Microbiology, 1986), "Characterization of cadmium susceptibility in *Mycobacterium avium*, *M. intracellulare* and *M. scrofulaceum*."
- Mayer, B.K. (M.S. Microbiology, 1985), "Investigation of catalase and superoxide dismutase from *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*".
- Fry, K. W. (M.S. Microbiology, 1984), "Comparison of environmental and clinical strains of *Mycobacterium avium*, *M. intracellulare* and *M. scrofulaceum*".

- Meissner, P.S. (Ph.D. Genetics, 1984), "Plasmids of *Mycobacterium avium*, *M. intracellulare* and *M. scrofulaceum*: isolation, use as epidemiological markers and role in heavy metal-resistance".
- Cosby, W.M. (M.S. Microbiology, 1984), "Genetic engineering lactobacilli for improved alfalfa ensiling and citrate resistance".
- Smith, R.T. (Ph.D. Microbiology, 1983), "Effect of temperature-induced membrane lipid phase transitions in recipient cells on conjugation in *Escherichia coli* K-12".
- Brooks, R.W. (M.S. Microbiology, 1983), "Soil as a possible origin of organisms of the *Mycobacterium avium*, *M. intracellulare* and *M. scrofulaceum* (MAIS) complex in the southeastern United States".
- Torres-Cabassa, A. S. (Ph.D. Genetics, 1982), "Genetic analysis and phenotypic characterization of Lon^- mutants of *Escherichia coli* K-12".
- Winfield, S. L. (M.S. Microbiology, 1979), "Genetic duplication in *Salmonella typhimurium*".
- Ferguson, K.P. (M.S. Microbiology, 1978), "Identification of a mutation affecting plasmid establishment in *Escherichia coli* K-12".
- Fiore, J. D. (M.S. Microbiology, 1978), "Studies of the utility of lectin-induced agglutination and bacteriophage adsorption in determining bacterial lipopolysaccharide composition".

TEACHING

Genetics - Sophomore introductory genetics course.

Genetics Laboratory - Sophomore laboratory course in genetic techniques.

Microbiology - Sophomore introduction to all aspects of microorganisms.

Microbial Genetics - A senior and graduate level course on the genetics of eukaryotic and prokaryotic microorganisms and their viruses.

Molecular Biology - A junior and senior level course on eukaryotic and prokaryotic molecular biology and recombinant DNA technology.

Topics in Microbial Genetics - Graduate, rotating topics course in microbial genetics.

Molecular Biology Laboratory - A senior and graduate level laboratory course in molecular biological techniques.

Humanities and the Biological Sciences - Course in the Humanities, Science, and Technology Program on issues surrounding genetic engineering.

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American Society for Microbiology
 Editorial Board, Applied and Environmental Microbiology
 (2002-present)
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 Member, Mycobacteriology Division Program Committee, 1989
 Member, Mycobacteriology Division Nominations Committee, 1988

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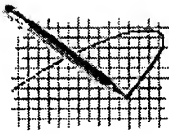
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 Chair, Microbiologist Search Committee, 1994-1995
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 Member, Graduate Examination Committee, 1984-1988
 Member, Research Committee, 1974-1976
 Member, Safety Committee, 1975-1990
 Member, Self Study Committee, 1975-1976
 Chair, Undergraduate Awards Committee, 1979-1980

JP010572

> Re: CSB Securitization
06/15/07 02:03 PM





Edward Purnell /JonesDay
Extension 5-4368
06/15/2007 01:43 PM

To Stacey N. Lefont/JonesDay@JonesDay
cc Glenn S. Arden/JonesDay@JonesDay, Sashay
Franklyn/JonesDay@JonesDay
bcc
Subject Re: CSB Securitization

Attached are proposed tax changes.

Ed



CSB.PDF

Edward A. Purnell
Jones Day
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Chicago, Illinois 60601-1692
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Stacey N. Lefont/JonesDay



Stacey N. Lefont/JonesDay
06/15/2007 10:29 AM

To Edward Purnell/JonesDay@JonesDay
cc Glenn S. Arden/JonesDay@JonesDay, Sashay
Franklyn/JonesDay@JonesDay
Subject Re: CSB Securitization

Ed,

I thought we had to limit the total number of holders of A's, B's, C's and D's to 99? (That is what I have in the document right now). We can probably limit the R to one holder only, if we need to. There is a sentence in the document now stating that there can be no more than one holder of the Class R Certificate, but the sentence is currently in brackets because I wasn't sure if it was necessary.

Also, please let me know if you have any comments to the forms of Certificates, which are included in the Exhibits document.

I think that our client is going to send out the drafts to Aegon today, but I can always send a subsequent set of changed pages reflecting tax comments.

Sashay Franklyn (one of our summer associates) is helping me on this transaction and can help process any changes you have in a new version of the document.

Stacey

Stacey N. Lefont
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Edward Purnell

From: Edward Purnell
Sent: 06/15/2007 11:16 AM EDT
To: Stacey Lefont
Cc: Glenn Arden
Subject: Re: CSB Securitization

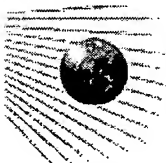
We need to limit transfers of the Cs, Ds and Rs (if the Rs are transferable). The As and Bs can be held without limitation. I'll have some minor changes to the pooling agreement later today.

Ed


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06/14/2007 07:03 PM

To: Edward Purnell/JonesDay@JonesDay
cc: Glenn S. Arden/JonesDay@JonesDay
Subject: Re: CSB Securitization 



Ed,

In your rider, what do the references to the Class E Certificates refer to? Should those references be to the Class R Certificate, which is held by the Seller and not freely transferable? Or should I delete the provisions relating to Class E? Or should I change all the references to Class C, D and E to Class A, B, C and D? (The A, B and C Certificates are the Senior Certificates, the D Certificates are the Junior Certificates and the R Certificate is issued to the Seller).

Stacey

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Facsimile: (212) 755-7306
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Edward Purnell/JonesDay



Edward Purnell /JonesDay
Extension 5-4368
06/14/2007 05:13 PM

To Stacey N. Lefont/JonesDay@JonesDay
cc Glenn S. Arden/JonesDay@JonesDay
Subject Re: CSB Securitization

The limitation language, attached below, should go in the pooling agreement -- probably in 7.04 (which will need some conforming changes). Any assignment or participation provision in the certificate purchase agreements should either incorporate the limit for the affected certificate (C or D) or cross reference the applicable pooling agreement limitation. There isn't any tax requirement that the limits be set out in the notes. You may want to cross-reference them in the notes nonetheless.

I've left blanks for the C, D and E sublimits. The sublimits must add up to 99 or less.



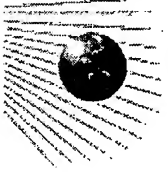
CHI_1593629_1_6_14 CSB SBA PSA Tax Rider.DOC

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Stacey N. Lefont/JonesDay



Stacey N. Lefont/JonesDay

06/14/2007 11:52 AM

To Edward Purnell/JonesDay@JonesDay, Glenn S.
Arden/JonesDay@JonesDay
cc

Subject Re: CSB Securitization

They are okay with the limit - I just need the language. Should the limitation be in the PSA or the Certificates themselves (or both)?

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Edward Purnell

From: Edward Purnell
Sent: 06/14/2007 11:51 AM CDT
To: Glenn Arden; Stacey Lefont
Subject: CSB Securitization

Could you get me the name of the tax person at Stroock? I'd like to discuss the need for a limit on the number of holders for the Cs, Ds and E .

Ed

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MANUAL OF CLINICAL MICROBIOLOGY

Third Edition

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**AMERICAN SOCIETY
FOR MICROBIOLOGY**

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Pref

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18. V
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20. G

Section V. Laboratory Tests in Chemotherapy

Chapter 41

General Considerations

JOHN C. SHERRIS AND JOHN A. WASHINGTON II

A number of considerations are involved in selecting an appropriate antimicrobial to treat an infection. (The term antimicrobial is used in this section to describe both antibiotics and chemotherapeutics.) These include: (i) knowledge of the inherent *in vitro* susceptibility of the infecting organism to appropriate antimicrobics; (ii) the relationship of the susceptibility of the strain to that of other members of the same species; (iii) pharmacological properties including toxicity, protein binding, distribution, absorption, and excretion, particularly under circumstances of existing or developing hepatic or renal failure; (iv) previous clinical experience of efficacy in treating infections due to the same species; (v) the nature of the underlying pathological process, its natural history, and its influence on chemotherapy; and (vi) the immune status of the host.

Of these factors, the concentrations of antimicrobial required to inhibit or kill organisms *in vitro* and those attained in body fluids during treatment are subject to direct measurement in the clinical laboratory. The purpose of this section of the manual is to provide detailed descriptions of appropriate procedures for these purposes. The susceptibility methods described are for use with bacteria other than mycobacteria, which are separately considered in Chapter 14.

The role of the laboratory in the selection and monitoring of chemotherapy was succinctly expressed by Theodore G. Anderson in the first edition of this manual: "When selecting an antimicrobial agent for therapy, it is the physician's responsibility to take into consideration the pharmacological characteristics of the several drugs as well as their relative antimicrobial effectiveness. The responsibility of the laboratory is to provide information through standardized *in vitro* tests, of the activity of appropriate antimicrobial agents against the organism in question." The methods given in the subsequent chapters constitute accepted approaches among the authors providing this information. Different procedures have been developed by others in a

number of countries, and the reader is referred to more detailed reviews for further information and for broader consideration of the theory of the subject (1, 2, 4, 5, 8, 10, 13-17).

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Influence of technical variation on susceptibility test results

The results of both dilution and diffusion susceptibility tests may be influenced markedly by the reagents and conditions of the tests, and these variables have been the source of considerable confusion in the past. Inoculum density is especially important. In addition, incubation time and temperature, pH, atmosphere, and stability of antimicrobics may all influence the endpoints obtained. Differences in constituents or ionic content of the medium, even between batches, may influence results of tests, particularly with the sulfonamides, tetracyclines, polymyxins, and aminoglycosides. In addition, diffusion tests are influenced by the growth rate of the organism and by the type, depth, and concentration of the agar used. For these reasons, special emphasis has been placed on reference procedures and methodological standardization (4, 6, 7, 11, 12, 19, 20), because only in this way can adequate reproducibility be obtained in investigative and clinical work.

In each of the susceptibility tests described, the inoculum is derived from several colonies. This is designed to reduce the chance of selecting variants derived from loss-mutations (e.g., loss of penicillinase production in staphylococci) or segregants from R-factor resistance markers. It also increases the chance of including representatives of the more resistant organism if more than one strain is represented by colonies that cannot be distinguished morphologically. The final inocula are reasonably heavy, which increases the chance of detecting high-frequency mutations to resistance and heteroresistant strains. The media selected show generally good

therapy

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SUSCEPTIBILITY

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buffering qualities and reproducibility and are of physiological pH. A central criterion of the conditions to be used in effective diffusion, dilution, or automated tests is that they must be able to detect strains carrying clinically important resistance determinants.

Selection of susceptibility tests methods

Diffusion test. The most widely used procedure is still the disk diffusion method, described in Chapter 44, which has been accepted by the Food and Drug Administration (FDA) (6, 7) and as a standard by the National Committee on Clinical Laboratory Standards (NCCLS) (11). This procedure, as normally used, is essentially a qualitative test which allocates organisms to the sensitive (susceptible), intermediate (or indeterminate), or resistant categories discussed below. The procedure is flexible in regard to the antimicrobics that can be tested, and it is easy to set up individual tests at different times. It is technically simple, although it requires careful attention to detail. It is generally applicable to organisms whose growth rate approximates those of the members of the *Enterobacteriaceae* family and *Staphylococcus aureus*, and the procedure has now also been adapted to detect penicillinase-producing strains of *Haemophilus influenzae* and *Neisseria gonorrhoeae*, and strains of pneumococci that have developed increased resistance to penicillin and some other antibiotics (see Chapter 44). In cases of clinical urgency, clinical material may serve as the inoculum for the test if the precautions indicated in Chapter 44 are followed. More experience has been gained over the years with this diffusion procedure than with any other test.

The deficiencies of the diffusion test are its nonquantitative interpretation, its inapplicability to many slow-growing organisms and anaerobes, and its inaccuracy in predicting susceptibility (as opposed to resistance) with antimicrobics, exemplified by the polymyxins, that diffuse poorly. Overall, however, it is an effective procedure for most routine tests but requires supplementation with a dilution test in situations when it is inapplicable or when more quantitative results are needed.

Dilution test. The most quantitative method for antimicrobial susceptibility testing is one of the dilution tests (considered in Chapters 42, 43, and 47) which are derived from the International Collaborative Study recommendations (4) or from proposed NCCLS standards. These yield direct quantitative results, are essentially uninfluenced by the growth rate of the organism, and avoid some of the complexities of diffusion properties of antimicrobics. Dilution tests do not

have the flexibility of the diffusion test, generally cannot be used for direct tests of clinical material because of the difficulty in detecting contamination, and, if reported quantitatively, require that the clinician be able to interpret the result or be helped in doing so.

The primary indication for dilution tests is to obtain quantitative susceptibility data when these are important or necessary for proper management of antimicrobial therapy. Although qualitative data are usually adequate for guiding the therapy of most infections, quantitative information may be needed when drug dosage schedules and serum levels must be closely monitored or under the conditions in which disk test results are inapplicable, equivocal, or unreliable. These conditions include tests on slow-growing organisms, confirmation of susceptibility (as opposed to resistance) to the polymyxins (B or E), confirmation of resistance to the aminoglycosides (particularly gentamicin, tobramycin, and amikacin), and tests with potentially toxic but clinically useful antimicrobics which yield immediately susceptible results by the disk test. Infections due to microorganisms which are categorized by the disk test as resistant or intermediate to the relatively nontoxic penicillins and cephalosporins may occasionally be treated preferentially and safely with large doses of one of these agents. Some urinary tract infections may respond to ordinary dosages of some antimicrobics because of the high levels which they attain in the urine. In these cases, the precise degree of susceptibility of an organism may influence the choice of antimicrobial, its dosage, and its route of administration. Other indications for dilution methods are for testing the susceptibilities of anaerobes by the methods described in Chapter 45 and for determining bactericidal activity or evidence of synergism or antagonism between antimicrobics against particular microorganisms. These procedures are considered in Chapter 46. Finally, dilution tests have been found to be practical and economical for routine purposes through the use of semiautomated microdilution techniques (see Chapter 43) or replica-plating agar dilution methods (see Chapter 42).

Any laboratory that intends to use the dilution test routinely and to prepare its own reagents and antibiotic dilutions must have the ability to prepare and maintain fully potent stock solutions of antimicrobial and to produce working dilutions on a regular basis. As with all susceptibility tests, the laboratory must control inoculum size and endpoint reading and must develop or use a quality control system that will give endpoints within the range of each series of antimicrobial dilutions. Recent data from profi-

ciency testing surveys show rather wide variation in quantitative results on the same strain tested in different laboratories by a variety of dilution procedures and schedules. Interlaboratory reproducibility is much better when well-controlled prediluted commercial systems are used, and similar improvement can be expected if the protocols used in Chapters 42 and 43 are followed.

Routine use of dilution procedures that do not use preprepared commercial plates is most appropriate for larger laboratories where there is sufficient skilled technical help to ensure reproducibility. Preprepared systems, when adequately controlled, are applicable to smaller laboratories.

Tests for antimicrobial inactivating enzymes. In some instances, resistant strains of bacteria among originally susceptible species owe their resistance exclusively to their ability to destroy or inactivate particular antibiotics. This is the case with penicillin and ampicillin resistance of strains of *Staphylococcus aureus*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae* that produce penicillinase. These can be detected rapidly and accurately with simple chemical procedures for detection of the enzyme (18), and results are available much more rapidly than those of orthodox susceptibility tests. Procedures for this purpose are described in Chapter 46.

Automated tests. Recently, a variety of mechanized or automated procedures have been developed for susceptibility testing, and these are considered in Chapter 48. Some facilitate the performance and reading of traditional overnight susceptibility tests. Others are designed to yield qualitative or limited quantitative results on the same day that the test is set up. Several of these procedures have already been evaluated by collaborative studies and have been shown to have a high degree of reproducibility. However, comparability of procedures providing rapid results with overnight dilution or diffusion tests has been more difficult to achieve with a few organism-antimicrobial combinations because the extent of inhibition of growth in the first few hours of contact may differ from that seen after overnight incubation. Most of these difficulties are being overcome by the use of heavier inocula or computer analyses of growth patterns in the presence of one or more concentrations of antimicrobial. These approaches are discussed in more detail in Chapter 48. Suffice it to say that automated methods have already been shown to have a place in routine work of larger laboratories when their limitations are recognized and avoided by using a traditional test on organism-antimicrobial combinations for which the automated test is inappropriate.

Interpretation of susceptibility tests: "susceptibility" and "resistance"

The interpretation of a quantitative susceptibility test result has three major components.

1. *The relationship of the MIC (minimal inhibitory concentration) or MLC (minimal lethal concentration) of the organism to the concentration of antimicrobial in the blood, or in some cases urine or other fluid, obtained with the dosage given.* This has proved a clinically useful approach, but is inevitably an incomplete model of the in vivo situation because of the varying degrees of protein binding, the interacting effects of host defense mechanisms, and the arbitrary aspects of the selection of test conditions.

2. *The relationship of the susceptibility of the strain under test to that of other members of the same species.* This is useful because the selection of resistant mutants or strains with extrachromosomal determinants of resistance has led to the appearance of populations of strains of some species well separated from the "wild" types that were previously uniformly susceptible to the antimicrobial. The resulting bimodal distribution of susceptibilities correlates well with clinical responsiveness. Thus, a strain falling in the more resistant population is considered a priori a resistant member of that species.

3. *Clinical experience with the treatment of the particular type of infection involved.* An ideal interpretation of susceptibility test results takes account of these factors independently. From a practical point of view, organisms are frequently allocated to predetermined "susceptible," "resistant," and one or more "intermediate" categories, and this approach was considered by the International Collaborative Study to continue to be useful and necessary in the light of presently available technical methods and general understanding of the principles of chemotherapy (4). The three categories recommended for the diffusion test given in Chapter 44 have been based on the synthesis of the first two criteria given above. They have been defined (11) as: (i) susceptible (or sensitive), implying that an infection due to the strain tested may be appropriately treated with the antimicrobics and dosages recommended for that type of infection and infecting species, unless otherwise contraindicated; (ii) resistant, containing strains not completely inhibited within the usual therapeutic dosage range; and (iii) intermediate, comprising a "buffer zone" which prevents major interpretative discrepancies that might result from small uncontrolled technical factors. The last category also includes strains which may respond to concentrations attainable by unusually high dosage or in areas, such as portions of the urinary tract, where the antimicrobial is concen-

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trated. The clinical extrapolations of these cat-
egories are, of course, subject to the considera-
tions given in the first paragraph of this chapter.

This three-category system requires qualifi-
cation in that it does not consider the blood
levels that may be attained with very high dos-
ages of the relatively nontoxic penicillins and
cephalosporins, the high urine levels of certain
antimicrobials, or the low blood levels achieved
with oral as opposed to parenteral dosage of
certain antimicrobics. Thus, it is important to
recognize the need for quantitative data in spe-
cial cases.

Indications for susceptibility tests in the clinical laboratory

Tests are indicated for organisms contributing
to the infectious process whose susceptibility
cannot be predicted from knowledge of their
identity. This applies, in particular, to *S. aureus*,
to gram-negative enteric organisms, to some an-
aerobes, and to unusual and opportunistic spe-
cies playing a pathogenic role. Specific patterns
of susceptibility (antibiogram) may be charac-
teristic of a species and may, therefore, assist in
correct species allocation. Antibiograms may be
determined for epidemiological reasons because
the occurrence of an unusual antibiogram for a
given species often assists in the recognition of
common-source outbreaks and patterns of cross-
infection.

Routine susceptibility tests are not needed
when resistance has not been described to the
antimicrobial of choice, e.g., *Streptococcus py-
ogenes* and *Neisseria meningitidis* to penicillin.
However, this situation needs to be reviewed
constantly, because the recent emergence of β -
lactamase-producing strains of *H. influenzae*
and *N. gonorrhoeae* (3) and of pneumococci
resistant to penicillin and other antibiotics (9)
has changed the requirements for testing among
these species. Susceptibility testing should be
avoided on members of the normal flora in their
normal habitat and on organisms that are known
not to be playing a pathogenic role. To make
such tests is both wasteful and misleading. The
routine diffusion susceptibility test described in
Chapter 44 should never be made on organisms
for which its interpretative criteria are inap-
plicable (e.g., slow growers or anaerobes).

Selection of antimicrobics for testing

For routine susceptibility testing, single rep-
resentatives of a group of related antimicrobics
are used whenever possible, and the FDA has
recognized specific class disks for many antimi-
crobic groups for use in the diffusion test (see
Chapter 44). However, there are sufficient dif-
ferences in the spectra of activity, or mecha-

nisms of resistance, among certain penicillins,
cephalosporins, and aminoglycosides that more
than one representative member of each of these
groups needs to be tested. The selection of an-
timicrobics to be tested should also be limited
to those that are clinically useful and appropri-
ate for the site of infection, except when the
procedure is being used to determine antibio-
grams for epidemiological purposes or when par-
ticular antimicrobics yield taxonomically useful
information. For example, tests with nitrofur-
antoin and nalidixic acid should be limited to
bacteria isolated from the urinary tract, and
tests of the combination of trimethoprim and
sulfamethoxazole should be limited to organisms
causing urinary tract infections or otitis media,
which are presently the only approved uses of
this combination in the United States. Tests
with methenamine mandelate should not be per-
formed because its in vivo activity depends on
urinary acidification to a pH of 5.0 or less, and
this condition is not reproduced in the ordinary
test systems.

The antimicrobics listed in Table 1 should
fulfill the basic requirements for routine tests in
most clinical laboratories, for aerobic and fac-
ultatively anaerobic bacteria, and the list conforms
closely to NCCLS recommendations. Additional
antimicrobics may be added as noted in the
footnotes to Table 1, when needed for special
problems of the individual patient, or to take
account of local preference or rules. Antimicro-
bics appropriate for testing anaerobes are con-
sidered in Chapter 45. To avoid confusion, re-
sults and tests on antimicrobics that are inap-
propriate in therapy and that are tested for
epidemiological or taxonomic purposes should
not be reported to the physician.

A decision to include a new antimicrobial in
the basic test set of a routine laboratory should
be based on (i) the use of the antimicrobial having
been approved by the FDA or its equivalent, (ii)
an activity spectrum differing significantly from
presently used analogs, (iii) readily available
information on its clinical pharmacology, (iv)
the availability of well-documented and FDA-
approved zone diameter interpretative criteria
in the case of disks for diffusion testing, and (v)
a clear clinical need for its introduction.

SPECIAL TESTS AND ASSAYS

Susceptibility tests make up the bulk of the
clinical laboratory tests which are ordered to
assist the clinician in his choice of chemothera-
peutics. They may need to be supplemented
with other procedures in certain complex clinical
situations, especially in subacute bacterial en-
docarditis and in severe infections in the immu-
nologically compromised. In these cases, deter-

TABLE 1.
Basic sets of antimicrobics to be tested routinely against rapidly growing aerobic and facultatively anaerobic bacteria^a

Antimicrobics	Staphylococci and streptococci	Enterococci	Enteric gram-negative bacilli other than <i>P. aeruginosa</i>		<i>P. aeruginosa</i>
			Urinary	Other	
Penicillins					
Ampicillin		1	1	1	
Carbenicillin			1	1	1
Nafcillin, oxacillin, or methicillin ^b	1				
Penicillin G	1	1			
Cephalosporins					
Cefamandole	2		1 ^c	1 ^c	
Cefoxitin	2		1 ^c	1 ^c	
Cephalothin	1		1	1	
Chloramphenicol	2	2		2	
Clindamycin	1				
Erythromycin	1	1			
Aminoglycosides					
Amikacin			1	1	1
Gentamicin	2		1	1	1
Kanamycin	2		1	1	
Tobramycin			1	1	1
Polymyxin B or E			2	2	1
Tetracycline	2	2	1	1	
Vancomycin	2				
Urinary tract agents					
Nalidixic acid			1		
Nitrofurantoin			1		
Sulfonamides			1		
Sulfamethoxazole/tri- methoprim			1		

^a 1 = Primary set; 2 = secondary drugs.

^b Oxacillin or nafcillin is preferable for detecting heteroresistant methicillin-resistant *S. aureus*.

^c Cefamandole and cefoxitin may be reserved for testing cephalothin-resistant organisms only.

mination of bactericidal concentrations or of the effect of combinations of antimicrobics may need to be measured. Direct tests of the ability of the antimicrobial in the patient's serum to inhibit or kill the infecting organism may also be helpful in monitoring the adequacy of dosage schedules (see Chapter 46). So far, there is still no general agreement on methods for determining minimal lethal (bactericidal) concentrations, studying antimicrobial combinations, or determining serum inhibitory or bactericidal activity against the infecting organisms. The procedures given should continue to serve as a basis for further studies towards methodological standardization of these important tests.

Increasingly, it is necessary to determine the amount of antimicrobial present in serum, urine, other fluids, or tissues. In clinical practice, this applies particularly to agents such as gentamicin, tobramycin, and amikacin, for which potentially toxic levels and therapeutic levels are very close. Serum assays are thus required to ensure that antimicrobial concentrations in the blood

are within a safe, but effective, range. This is particularly the case in patients with renal deficit, in whom serum levels of antimicrobial may be less predictable. Simple, rapid, and accurate methods for this procedure are given in Chapter 47.

FUTURE NEEDS

Since the last edition of this manual, an NCCLS standard for diffusion susceptibility tests has been published and an updated revision has recently appeared (11). A standard for anaerobic susceptibility testing (12) has also been published, and one for dilution tests is in the final stages of preparation. These provide reference points for clinical, epidemiological, and research studies. Concurrently, quality control values for standard strains have been developed and updated to include new antimicrobics. Major proficiency testing programs for susceptibility test results are now operating through the Center for Disease Control and the College of American Pathologists. Thus, the background

for improvement of susceptibility test procedures to give greater accuracy and better inter-laboratory reproducibility has been laid. It is especially important that mechanisms be maintained for regular updating of reference procedures, interpretative standards for new antimicrobics, recommendations for basic routine sets of antimicrobics for testing, and data on performance of quality control strains. Supplements providing such data are planned by the NCCLS.

Developments in mechanization and commercially available test kits have now brought the capacity for quantitative susceptibility testing even to small laboratories, and this trend is likely to continue. Automated procedures that yield rapid results and can be directly interfaced with computer reporting systems are already available, and more can be expected to be developed. There is a clear need for agreement on evaluative protocols by which the relationship between the performance of new systems and reference procedures can be assessed and for definitions of acceptable performance. Potential purchasers need access to such data before deciding whether to incorporate a system into their routine work.

Media used for susceptibility testing remain a problem. Performance standards for commercially produced Mueller-Hinton media are needed for application at the manufacturer's level. For instance, it has become increasingly apparent that there are variations in performance of *Pseudomonas aeruginosa* with the newer aminoglycoside antimicrobics when tested with different methods and with batches of media from the same or different manufacturers, resulting in MICs ranging over several dilution steps. This has caused problems of interpretation that can only be resolved by performance control at the manufacturer's level. With the wider acceptance of reference procedures, there is need for a review of qualitative interpretative categories used in diffusion and in some dilution and automated procedures. The boundaries of interpretative categories have involved some best-judgement types of decisions and subjective clinical experience. Moreover, validation of categories by detailed clinical studies has been made difficult in the past by inadequate methodological standardization. This should now be possible, particularly with new antimicrobics.

Methodological agreement is needed on techniques for determining bactericidal endpoints, for measurements of the effects of combinations, and for performing serum inhibitory or bactericidal tests. In the absence of standardized methods or reference procedures, results from different laboratories cannot be compared with con-

fidence, and an adequate base of experience has not been developed for fully satisfactory interpretation of the results. For all of these procedures, the kinetics of microbial killing by antimicrobics make it essential that statistical endpoints be accepted, such as those recommended in Chapter 46.

In summary, we may reasonably look forward to improved performance of media and of procedures for orthodox susceptibility tests, to improvements in selecting and disseminating interpretative recommendations, and to improved quality control. Beyond that, rapid automated or semiautomated procedures for susceptibility testing are coming into use and should result in better reproducibility through elimination of many sources of technical error. There continues to be a need for reference or agreed procedures for determining lethal endpoints, interactions of combinations, and bactericidal activities of serum so that interpretations of the results of these tests can be refined through cumulative experience. These developments should increase the usefulness of laboratory procedures in the selection and monitoring of chemotherapy.

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Dilu

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CONCENTRATION

For most tests, a 0.5 ml is a standard volume with any tests of *Pseudomonas* 100 to 120% inhibitory concentration whose concentration Under these conditions will need tests of 10% limits of concentration will vary considerably, how low the inhibitory concentration, response to systemic dosage (Collaborative) trations appropriate standard.

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DIAGNOSTIC MICROBIOLOGY

A textbook for the isolation and
identification of pathogenic
microorganisms

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FOURTH EDITION

with 92 illustrations

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PREP

FOURTH EDITION

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34 Determination of susceptibility of bacteria to antimicrobial agents

According to Isenberg,¹² the value of the clinical laboratory can be measured only by the significance of the guidance it gives the practicing physician in the treatment of his patients. In no other area of clinical microbiology does this statement become more pertinent than in the testing of clinical isolates for their susceptibility to antimicrobial agents. With the increasing number of these agents at the physician's disposal and the changing pattern of resistance and susceptibility among bacteria—particularly the gram-negative enteric bacilli—the clinician must rely more and more upon sensitivity testing to guide his selection of appropriate drugs or alter an already imposed regimen. Therefore, to a large extent, a laboratory report showing susceptibility or resistance to a particular antimicrobial agent becomes an endorsement of its usefulness or withdrawal.

Since the microbiologist must become an adviser to the physician regarding proper antimicrobial therapy, it follows, therefore, that he must maintain (1) a high level of accuracy in his testing procedures, (2) a high degree of reproducibility for the results, and (3) a good correlation of his results with the clinical response.¹² Only through close cooperation and exchange of information between the laboratory staff and the clinician can the best possible management of an infectious process be achieved.

The principal methods presently used

by the laboratory to determine susceptibility of a microorganism to an antibiotic include the **dilution tests**, such as the broth tube and agar plate dilution procedures, and the **agar diffusion test**, utilizing antibiotic-impregnated discs. Each method has its advantages and its limitations, and these must be understood and appreciated in order to obtain maximum usefulness of the results. Since there is a place for all of these methods in the clinical laboratory, the procedures and directions for the use of each will be described in detail.

In the interpretation of any in vitro susceptibility tests, it is well to remember that they are essentially **artificial measurements**; the data yielded by them give only the approximate range of effective inhibitory action against the microorganisms. The only absolute criterion of microbial response to antibiotics is the **clinical response** of the patient when adequate dosage of the appropriate antibiotic is administered.

BROTH TUBE DILUTION METHOD FOR DETERMINING SUSCEPTIBILITY TO ANTIBIOTICS

In the broth tube dilution method for determining the susceptibility of an organism to antibiotics, specific amounts of the antibiotic, prepared in decreasing concentration in broth by the serial dilution technique, are inoculated with a culture of the bacterium to be tested. The susceptibility

of the organisms is determined, after a suitable period of incubation, by microscopic observation of the presence or absence of growth in the varying concentrations of the antimicrobial agent. This bacteriostatic end-point value is known as the **minimal inhibitory concentration (MIC)**. With minor additions, the technique can be adapted to the determination of bactericidal levels of the antibiotic—the **minimal bactericidal concentration (MBC)**; this is discussed at length in a further section.

A number of factors must be considered in establishing the procedures and in evaluating the results of these tests.⁸ They include the following: (1) the medium in which the tests are performed, (2) the stability of the antibiotic, (3) the size of the inoculum, (4) the rate of growth of the organism, and (5) the period of incubation of the tests. Any variation in one or more of these factors may influence the tests, and the results obtained by one procedure may not agree with those arrived at by a slightly different method.⁵ However, if a **standard procedure using only pure cultures** is adopted and strictly adhered to, reproducible results can usually be obtained and the reports from a given laboratory can be readily interpreted by the clinical staff.

The test tube serial dilution method gives a fairly accurate determination of susceptibility to measured amounts (either units or micrograms) of the antibiotic. It is a time-consuming and expensive procedure, however, especially when the clinician wants to know the susceptibility of an organism to a number of antibiotics. For this reason its use may well be restricted to special cases when quantitative results may be of value. In any event, it is strongly recommended that all clinical laboratories should be prepared to offer this service to the clinician, either directly or through a referral laboratory.

The serial dilution method may be recommended for determining the susceptibility of organisms isolated in the following instances: (1) from blood cultures, (2)

from patients who fail to respond to apparently adequate therapy, and (3) from patients who relapse while undergoing such therapy. The study of organisms from the third instance usually involves determination of any increase in resistance of subsequent isolations and requires special methods.

Routine procedure for serial dilution tests

Preparation of stock solution of antibiotics

Stock solutions of antibiotics are prepared from concentrated, dehydrated sterile material of known potency that may be obtained from the pharmaceutical manufacturer. Generally, they are prepared in concentrations of 1,000 µg./ml., using phosphate buffer or Mueller-Hinton broth as the diluent and are tubed in 1-ml. amounts in screw-capped vials.

When stored in the frozen state at -20° C., these antibiotics will remain stable for at least 8 weeks*; when refrigerated at 5° C., they show no appreciable loss of potency in 1 week. Any unused thawed solutions of antibiotics should be discarded; each aliquot should be sufficient for 1 day's use only and should not be refrozen.

Table 34-1 is provided as a guide to the preparation of stock solutions of the most frequently used antimicrobial agents.

Selection of media

The fluid media in which the tube dilution sensitivity tests are carried out must be the kind that will support optimal, rapid growth of the test organism in pure culture. A broth medium that will support the growth of pneumococci and streptococci without the addition of serum or blood is preferable, since the addition of such enrichment adds another variable to the test and may influence the results. Trypticase soy broth, or preferably Mueller-Hinton broth† is recommended for sensitivity tests with the following ex-

*Ampicillin requires storage at -60° to -70° C. to prevent loss of potency.

†This medium appears to be low in tetracycline and sulfonamide inhibitors and shows good batch-to-batch consistency.

Table 34-1. Procedures for preparing stock solutions*

ANTIBIOTIC	MANUFACTURER	METHOD OF PREPARATION
Ampicillin	Bristol-Myers Co.	Weigh out material and multiply by "activity standard" provided by manufacturer.† Add 0.1 ml. of pH 8.0 phosphate buffer to dissolve; dilute with pH 6.0 phosphate buffer.
Penicillin G	Eli Lilly & Co.	Add 60 ml. of water to a vial containing 1 million units or 600,000 µg.; this makes a stock solution of 10,000 µg./ml.
Methicillin	Bristol-Myers Co.	Weigh out material and multiply by "activity standard" from manufacturer; dilute with pH 6.0 phosphate buffer.
Oxacillin	Bristol-Myers Co.	Add 16 ml. of water to a vial to give 1,000 µg./ml.
Cephalothin	Eli Lilly & Co.	Weigh out material and multiply by the "activity standard" from the manufacturer; dilute with pH 6.0 phosphate buffer.
Cephalexin	Eli Lilly & Co.	Weigh out exactly 30 mg., add 30 ml. of pH 6.0 phosphate buffer to give 1,000 µg./ml.
Carbenicillin	Beecham-Massengill Pharmaceuticals	Add 10 ml. of water to a vial containing 1 gm. of drug, then dilute 1:100 to give 1,000 µg./ml.
Tetracycline	Pfizer Lab.	Add 20 ml. of water to a vial that contains 20 mg. of drug, to give 1,000 µg./ml.
Chloramphenicol	Parke, Davis & Co.	Weigh out exactly 50 mg., add 1 ml. of ethyl alcohol to dissolve drug and sufficient water to 50 ml. This gives a concentration of 1,000 µg./ml.
Erythromycin	Abbott Laboratories	Weigh out material and multiply by the "activity standard" from the manufacturer.† Dissolve in 1-2 ml. alcohol and add water to a final concentration of 1,000 µg./ml.
Lincomycin	The Upjohn Co.	Add 20 ml. water to a vial to make 1,000 µg./ml.
Clindamycin	The Upjohn Co.	Add 15 ml. of water to vial containing 150 mg. of drug, dilute 1:10 to give 1,000 µg./ml.
Kanamycin	Bristol-Myers Co.	Weigh out exactly 30 mg., add 30 ml. water to give 1,000 µg./ml. Agent is unstable in acid range.
Gentamicin	Schering Corp.	Weigh out material and multiply by "activity standard" from manufacturer, † dilute with water to give 1,000 µg./ml.
Polymyxin B	Burroughs Wellcome & Co.	Add 5 ml. water to a vial containing 50 µg., to give 10,000 µg./ml.; dilute to desired concentration.
Colistin (polymyxin E)	Warner-Chilcott Lab.	Weigh out material and multiply by "activity standard" from manufacturer, † dilute with water to desired concentration.
Bacitracin	The Upjohn Co.	Add 10 ml. of water to a vial containing 10,000 units, to give 1,000 units/ml.
Nitrofurantoin	Eaton Labs., Inc.	Weigh out 120 mg. of drug and transfer to a 50-ml. flask containing 4.0 ml. of dimethyl formamide. Heat in 56°C. water bath with shaking to dissolve. This solution contains 30 mg./ml.
Nalidixic acid	Winthrop Labs.	Weigh out approx. 30 mg., add 2 ml. of 1 N NaOH and allow to stand to dissolve (may require gentle heat). Dilute with sterile water (less 2 ml.) to desired concentration.

*Courtesy of John A. Washington II, Head, Section of Clinical Microbiology, Mayo Clinic.

†Example: 1 mg. = 825 µg. ("activity standard"/µg.). 50 mg. = 50 × 825 = 41,250 µg. Therefore, add 41.2 ml. of diluent to give 1,000 µg./ml.

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ceptions: microaerophilic streptococci, which are frequently isolated from cases of subacute bacterial endocarditis, strictly anaerobic streptococci, *Bacteroides* species, and clostridia should be tested in fluid thioglycollate medium enriched with hemin and vitamin K.; some strains may require additional enrichments to support good growth. In the case of all fastidious organisms the growth requirements should be determined before sensitivity tests are carried out, in order that the fluid medium supporting the most luxuriant and rapid growth may be selected for the procedure. Hemoglobinophilic organisms, such as *Haemophilus* species, must necessarily be tested in Mueller-Hinton broth containing 1% rabbit blood. The blood may be added to the broth before it is distributed into the test tubes, or it may be added with the inoculum.

Procedure for preparing serial dilutions and determining susceptibility

1. Thaw the frozen stock solution of the antibiotic(s) required and dilute 1:5 with sterile Mueller-Hinton broth. This gives a working solution containing 200 µg. or units each. For bacitracin a concentration of 100 units per ml. may be used.
2. Select 10 clear, sterile, cotton-

plugged or capped test tubes of small size (13 × 100 mm.) and mark from 1 to 10.

3. Using aseptic technique, pipette 0.5 ml. of dilution broth into tubes 2 through 10. Do this for each antibiotic to be tested.
4. Add 0.5 ml. of the working solution (200 µg./ml.) of the antibiotic into tubes 1 and 2. Mix contents of the second tube well and transfer 0.5 ml. to tube 3. Mix well and transfer 0.5 ml. to tube 4, continuing this procedure to tube 9. Discard 0.5 ml. from tube 9; the tenth tube receives no antibiotic and serves as the control. Use a separate pipet for each transfer to avoid any carry-over.
5. To all tubes add 0.5 ml. of an inoculum containing approximately 10^5 to 10^6 organisms per milliliter. This may be prepared in most instances by making a 1:1,000 dilution in broth of an overnight (6-hour if a rapidly growing organism) broth culture of the organism to be tested. With slow-growing organisms, such as microaerophilic streptococci, *Bacteroides*, and so forth, it may be necessary to use cultures in thioglycollate medium up to 48 hours old. If numerous antibiotics are to be tested,

Table 34-2. Antibiotic serial dilution—tube setup

TUBE	DILUENT (MEDIUM) ADDED (ML.)	ANTIBIOTIC ADDED	DILUTED CULTURE ADDED (ML.)	FINAL ANTIBIOTIC CONCENTRATION	
				ALL BUT BACITRACIN (UNITS OR µG.)	BACITRACIN (UNITS)
1	None	0.5 ml. working solution	0.5	100	50
2	0.5	0.5 ml. working solution	0.5	50	25
3	0.5	0.5 ml. from tube 2	0.5	25	12.5
4	0.5	0.5 ml. from tube 3	0.5	12.5	6.25
5	0.5	0.5 ml. from tube 4	0.5	6.25	3.125
6	0.5	0.5 ml. from tube 5	0.5	3.125	1.56
7	0.5	0.5 ml. from tube 6	0.5	1.56	0.78
8	0.5	0.5 ml. from tube 7	0.5	0.78	0.39
9	0.5	0.5 ml. from tube 8*	0.5	0.39	0.19
10	0.5	None	0.5	Zero	Zero

*Discard 0.5 ml. from tube 9.

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IC CONCENTRATION

BACITRACIN (UNITS)

50
25
12.5
6.25
3.125
1.56
0.78
0.39
0.19
Zero

prepare sufficient inoculum in a flask,
for uniformity.

The final volume in each tube is
now 1 ml., and the antibiotic range
covered in the series is from 100 μ g.
or units to 0.39 μ g. or unit per milli-
liter, in twofold steps (Table 34-2).

For organisms highly susceptible
to antibiotics, such as streptococci
and pneumococci, a lower range of
antibiotic dilutions may be employed
by further diluting the working solu-
tion (200 μ g. or units per milliliter)
1:10. Then, by the serial twofold di-
lution technique shown in Table
34-2, the final concentrations will be
10, 5, 2.5, 1.25, 0.63, 0.3, 0.15, 0.08
and 0.04 μ g. or units per milliliter.

6. Incubate the series at 36° C. and ex-
amine macroscopically for evidence
of growth. Incubate the tubes only as
long as it is necessary for the control
tube to show turbid growth; usually
12 to 18 hours is the optimal time.
The last tube, that is, the lowest con-
centration of the antibiotic in the
series showing no growth, is taken
as the MIC of the antibiotic and is
expressed as micrograms (or units)
per milliliter. It is well to remember
that in the serial dilution technique
there is a possible error equivalent to
one tube dilution, so that the MIC
values are not necessarily actual val-
ues but are near true values.

To determine the MBC, pipette 0.5
ml. of each tube of the tube dilution
set that shows no visible turbidity
into 12 ml. of infusion agar, mix, and
make a pour plate. Having obtained
a colony count of the initial inocu-
lum by making a pour plate of the
1:1,000 dilution when setting up the
test, one may then calculate the
lowest concentration of the antimi-
crobial that provided a 99.9% and
100% bactericidal activity, by com-
paring colony counts, after an appro-
priate incubation period of 48 to 72
hours.

AGAR PLATE DILUTION METHOD FOR DETERMINING SUSCEPTIBILITY TO ANTIBIOTICS

The agar plate dilution method¹ is simi-
lar in principle to the tube dilution meth-
od, except that a solid medium is used.
Mueller-Hinton agar is recommended and
is prepared in 100-ml. amounts. Some
workers incorporate 5% blood or heated
blood in the medium when using it for or-
ganisms that require enriched media, such
as pneumococci, streptococci, and *Haemo-*
philus. There appears to be no significant
inactivation of the antibiotics by the addi-
tion of the blood.

Procedure for preparing serial dilutions

Prepare twofold serial dilutions* of the
stock antibiotics, as described in the pre-
vious section, using at least ten times the
volumes indicated. Stock solutions con-
taining 1,000 μ g. per milliliter are most
useful, since decimal dilutions are readily
prepared from these. For example, to
prepare a 10 μ g. per milliliter plate, add 1
ml. of the stock to 100 ml. of melted and
cooled agar and pour plates of the same.

Preparation of plate dilutions

Melt and cool sufficient screw-capped
flasks of agar medium for the number of
plates to be prepared (about 20 ml. of me-
dium is required per 90 mm. diameter
plate) and allow to equilibrate in a water
bath at 50° before adding the antibiotic.
Add the required amount of the various
antibiotic dilutions to each flask, mix gen-
tly by inversion, and pour into plates.[†]
Allow the agar to harden and store in the
refrigerator at 5° C. until used, preferably
within 24 hours (and not after 1 week) of
preparation.[‡]

*Some workers prefer final dilutions to contain 20,
10, 5, 1, 0.1, and 0.01 μ g. per milliliter of medium.

†It is not recommended that the antibiotic dilutions
and culture medium be mixed directly in the plates;
this may produce uneven distribution of the antibiot-
ic in the agar.

‡Media containing unstable antibiotics, such as am-
picillin, should be prepared twice weekly.

Inoculation of plates

The inoculum size should be adjusted to contain approximately 10^8 organisms per milliliter (equivalent to a McFarland standard of 1 or 2); this will ensure dense, nearly confluent growth on a control plate containing no antibiotic.

Spot inoculation of the plates is made with a 1-mm. loop (approximately 0.001 ml.), a capillary pipet, or, preferably, by using the inocula replicator of Steers and co-workers.¹⁷ In this device, each single manipulation will release thirty-six different cultures from the prongs on a replicator head to the surface of a 100×15 mm. square plastic plate (Falcon) containing agar to a depth of 3.0 mm. Each prong will deliver about 0.001 ml.; thirty-six inoculations can thus be made simultaneously.

In using the Steers replicating device, it is recommended that one space on each plate be allocated to a marking solution (for proper orientation of the plate), one space for testing the viability of the test strain, and two spaces for controls—strains of gram-positive cocci and gram-negative bacilli of known stable MIC's to the antibiotics used. Thus, 32 spaces will then be available per plate for the testing of clinical isolates.

Organisms having a spreading tendency, such as *Proteus* and *Pseudomonas*, may be contained by the use of glass cylinders,* as suggested by Washington.²¹

Incubation and reading of tests

Incubate the plates at 36° C. for 16 to 18 hours and examine for the presence of growth. The lowest concentration of the antibiotic producing **complete inhibition of growth**† is taken as the end point. Partial inhibition can be observed readily by noting the gradual decrease in amount of growth until complete inhibition is ob-

tained. The control cultures on antibiotic-free media should show confluent growth.

STANDARDIZED DISC-AGAR DIFFUSION METHOD FOR DETERMINING SUSCEPTIBILITY TO ANTIBIOTICS

Perhaps the most useful, and certainly the most used, laboratory test for antibiotic susceptibility is the antibiotic disc-agar diffusion procedure, usually called the **disc method**. Its simplicity, speed of performance, economy, and reproducibility (under standardized conditions) makes it ideally suitable for the busy diagnostic laboratory when the more laborious dilution methods previously described may not be practiced.

In this method, as originally described by Bondi and associates,⁴ filter paper discs that have been impregnated with various antimicrobial agents of specific concentrations are carefully placed on an agar culture plate that has been inoculated with a culture of the bacterium to be tested. The plate is incubated overnight and observed the following morning for a **zone of growth inhibition** around the disc containing the agent to which the organism is **susceptible**, whereas a **resistant** organism will grow up to (and under) the periphery of the disc.

No attempt will be made here to discuss the complex physicochemical reactions that take place during the diffusion of the antibiotic into the agar gel or the dynamics of bacterial growth on the substrate—the reader is referred to publications by Ericson^{6,7} for details concerning these.

In recent years numerous attempts have been made to standardize the disc procedure, including the work of Bauer, Kirby, and co-workers,³ Ericsson,⁷ the World Health Organization (WHO), the Food and Drug Administration,⁹ and most recently, the National Committee for Clinical Laboratory Standards (NCCLS). Because the revised *Tentative Standards*¹⁴ recommended by the Subcommittee on Antimicrobial Susceptibility Testing of the NCCLS appears to be the most explicit in

*12 × 12 mm. Raschig rings, Scientific Glass Apparatus Co., Bloomfield, N. J.

†A very fine growth or a few visible colonies also may occur when the Steers replicator is used; this may be disregarded in the reading of the test.

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methodology, these recommendations are the ones that will be presented herein.

Numerous proficiency testing surveys, including a recent nationwide laboratory evaluation by the Center for Disease Control (CDC)¹¹ of the disc procedure have revealed that (1) the procedure as practiced is not standardized and (2) there are numerous variables that may contribute to these discrepancies. Among those which have been identified are the following:

1. Selection and concentration of antimicrobial discs
2. Selection, volume, and age of plating medium
3. Storage and handling of discs
4. Methodology of testing
5. Criteria used for interpreting results

Selection of antimicrobial discs

The following basic set of drugs* and their concentrations are recommended for routine susceptibility testing:

Ampicillin	10 µg.
Bacitracin	10 U.
Carbenicillin†	100 µg.
Cephaloridine	30 µg.
Cephalothin	30 µg.
Chloramphenicol	30 µg.
Clindamycin	2 µg.
Colistin (Polymyxin E)	10 µg.
Doxycycline	30 µg.
Erythromycin	15 µg.
Gentamicin	10 µg.
Kanamycin	30 µg.
Lincomycin	2 µg.
Methicillin	5 µg.
Nafcillin and oxacillin	1 µg.
Nalidixic acid	30 µg.
Neomycin	30 µg.
Nitrofurantoin	300 µg.
Penicillin G	10 U.
Polymyxin B	300 U.
Streptomycin	10 µg.
Sulfonamides	300 µg.
Tetracycline	30 µg.
Vancomycin	30 µg.

A basic set of discs for routine testing against the commonly isolated microorganisms is listed on p. 322.

*Available from Baltimore Biological Laboratory, Cockeysville, Md.; Difco Laboratories, Detroit; Pfizer Diagnostics, Flushing, N. Y.; and others.

Selection of plating medium

Although an ideal medium has not yet been perfected for the disc test, the NCCLS Subcommittee considers Mueller-Hinton agar the best compromise for routine susceptibility testing, since it shows good batch-to-batch uniformity and is low in tetracycline and sulfonamide inhibitors. With the addition of 5% defibrinated sheep, horse, or other animal blood, it will support the growth of the more fastidious pathogens that will not grow on the non-enriched medium. When required, the blood-containing medium may be "chocolatized," for testing *Haemophilus* species.

Mueller-Hinton agar* is prepared according to the manufacturer's directions and should be immediately cooled in a 50° C. water bath after removal from the autoclave. This is then poured into sterile dishes (on a level, horizontal surface) to a uniform depth of 4 mm.; this is equivalent to approximately 60 ml. in a 140-mm. (internal diameter) plate, or approximately 25 ml. for 90-mm. plates. After cooling at room temperature, the plates may be used the same day, or stored in the refrigerator at 2° to 8° C. for not more than 7 days, unless some method of minimizing water loss from evaporation is taken.† As a sterility control, several plates from each batch of blood-containing Mueller-Hinton agar should be incubated at 36° C. for 24 hours or longer but not used subsequently.

Each batch of Mueller-Hinton agar should be checked for pH when prepared; it should be pH 7.2 to 7.4 at room temperature. This may be tested by macerating a small amount of the medium in a little distilled water, or by allowing a little of the medium in a small beaker to gel around the pH meter electrode,‡ and reading the pH.

*Available from Baltimore Biological Laboratory, Cockeysville, Md.; Difco Laboratories, Detroit; and others.

†Such as wrapping in polystyrene plastic.

‡If available, a surface electrode is desirable.

Just before the medium is used, the plates should be placed in a 36° C. incubator with lids partly ajar, until excess surface moisture has evaporated—usually requiring 10 minutes.

Storage and handling of discs

Antibiotic susceptibility test discs are generally supplied in separate containers with a desiccant* and should be kept under refrigeration (below 10° C.). Discs containing the penicillins (including ampicillin and carbenicillin) and cephalosporin drugs should always be kept frozen (at less than -14° C.) to maintain their potency; a small working supply may be refrigerated for up to 1 week. For long-term storage, discs are best kept in the frozen state until needed.

As they are required, the unopened containers are removed from the refrigerator or freezer 1 or 2 hours before the discs are to be used and allowed to adjust to room temperature, in order to minimize condensation resulting from warm air reaching the cold containers. If disc dispensers are utilized, they should be equipped with tight covers and supplied with a satisfactory desiccant; when not in use, they should also be refrigerated.

Manufacturer's expiration dates should be noted and listed; discs must be discarded on their expiration date.

Preparation of inoculum

It has been shown by various workers that when certified antibiotic discs and a single standard culture medium are used, the greatest factor contributing to reproducibility of the disc test is the control of the inoculum size.

The currently recommended method of preparing a standardized inoculum is as follows:

1. With a wire loop, the tops of four or five isolated colonies of a similar morphological type are transferred to

a tube containing 4 to 5 ml. of soybean-casein digest broth.*

2. The broth is incubated at 36° C. until its turbidity exceeds that of the standard (described in step 3). This usually requires 2 to 5 hours' incubation.
3. The turbidity is then adjusted to a barium sulfate standard that is prepared by adding 0.5 ml. of 1.175% w/v barium chloride hydrate ($\text{BaCl}_2 \cdot 2 \text{H}_2\text{O}$) to 99.5 ml. of 1% v/v (0.36N) sulfuric acid. The standard is distributed in screw-capped tubes of the same size as the ones used in the broth culture, approximately 4 to 6 ml. per tube, which are then tightly sealed and stored at room temperature in the dark. Fresh standards must be prepared at least once every 6 months, although a recent publication suggests that the solution remains stable for a much longer period when heat-sealed and stored in the dark.²²
4. The barium sulfate standard must be vigorously agitated in a Vortex shaker just before use, and the turbidity of the broth culture is then adjusted visually by adding sterile saline or broth, using an adequate light and comparing the tubes against a white background with a contrasting black line.

Inoculation of the test plates

Within 15 minutes of adjusting the density of the inoculum, a sterile cotton swab on a wooden applicator stick (plastic sticks are not satisfactory) is dipped into the standardized bacterial suspension and the excess fluid is removed by pressing against the inside of the tube above the fluid level. The swab is then used to streak the dried surface of a Mueller-Hinton plate in several planes (by rotating the

*Humidity, particularly high humidity, heat, and contamination are important deteriorating factors.¹⁰

*Trypticase soy broth, Baltimore Biological Laboratory, Cockeysville, Md.; tryptic soy broth, Difco Laboratories, Detroit; and others.

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adjusting the den- sterile cotton swab ick (plastic sticks dipped into the pension and the d by pressing tube above the en used to streak Mueller-Hinton by rotating the

plate approximately 60° each time) to en- sure an even distribution of the inoculum.

Allow the inoculated plates to remain on a flat and level surface undisturbed for 3 to 5 minutes to allow for adsorption of excess moisture, then apply the discs, as described in the following section.

Placement of discs

With alcohol-flamed, fine-pointed forceps (cooled before using) or a disc dispenser,* the selected discs are placed on the inoculated plate and pressed firmly into the agar with a sterile forceps or needle, to ensure complete contact with the agar. The discs are distributed evenly in such a manner as to be no closer than 15 mm. from the edge of the petri dish and so that no two discs are closer than 24 mm. from center to center. Once a disc has been placed, it should not be moved, since some diffusion of the antibiotic occurs almost instantaneously.

An alternative method, using an agar overlay, has been described by Barry and colleagues.² This method is useful only for rapidly-growing organisms such as *Staphylococcus aureus*, the enteric bacilli, and *Pseudomonas aeruginosa*, and must be standardized to correspond with results obtained by the cotton swab-streak method already described.

The inoculated and disced plates are inverted and placed in the 36° C. incubator within 15 minutes after application of the discs. Incubation under increased CO₂ tension should not be practiced, since the interpretative zone sizes were developed under aerobic conditions; furthermore, CO₂ incubation may significantly alter the zone sizes.

Table 34-3 is presented as a practical guide in the selection of discs for routine susceptibility testing of facultative organisms isolated in clinical practice. Although

not identical to that recommended by the NCCLS,¹⁴ it has proved of value to clinicians at the Wilmington Medical Center, and has served as an aid in reducing the misuse or overuse of antibiotic agents in a large medical complex.

Reading of results

After incubation the relative susceptibility of the organism to the antibiotic is demonstrated by a clear zone of growth inhibition around the disc. This is the result of two processes: (1) diffusion of the antibiotic and (2) growth of the bacteria. As the antibiotic diffuses through the agar medium from the edge of the disc, its concentration progressively diminishes to a point where it is no longer inhibitory for the organism, which then grows freely. The size of this area of suppressed growth, the **zone of inhibition**, is determined by the concentration of the antibiotic present in the area. Therefore, within the limitations of the test, the **diameter of the inhibition zone** denotes the **relative susceptibility** to a particular antibiotic.

After 16 to 18 hours' incubation,* each plate is examined and the diameters of the complete inhibition zones are noted and measured, using reflected light and sliding calipers, a ruler, or a template prepared for this purpose and held on the bottom of the plate. The **end point**, measured to the nearest millimeter, should be taken as the area showing no visible growth that could be detected with the unaided eye. Faint growth or tiny colonies near the edge of the inhibition zones are ignored, as is the veil of swarming occurring in the inhibition zones of some strains of *Proteus* species. With sulfonamides, slight growth (with 80% or more of inhibition) is disregarded, and the margin of heavy growth is measured to determine the zone diameter.

Large colonies growing in an inhibition

*Dispensers for both the 90-mm. and 140-mm. Petri plates are available from Baltimore Biological Laboratory, Cockeysville, Md.; Difco Laboratories, Detroit; Pfizer Diagnostics, Flushing, N. Y.; and others.

*Microbial growth should be almost or just confluent; if only isolated colonies are present, the inoculum was too light and the test must be repeated.

Table 34-3. Schema for recommended antimicrobial discs*

ORGANISM	AM 10 µg.	CB 50 µg.	CF 30 µg.	CM 30 µg.	CC 2 µg.	EM 15 µg.	GM 10 µg.	KM 30 µg.	LN 2 µg.	DP 5 µg.	NA 30 µg.	NF 300 µg.	PN 10 u.	PB 300 u.	SM 10 µg.	TE 30 µg.
Gram-negative rods																
<i>Escherichia coli</i>	x	[x]	x	[x]			x	x			(x)	(x)		x	[x]	[x]
<i>Klebsiella-Enterobacter-Serratia</i>	x		x	[x]			x	x			(x)	(x)		x	[x]	[x]
Other enteric bacilli	x		x	[x]			x	x			(x)	(x)		x	[x]	[x]
<i>Proteus</i> species	x	[x]		[x]			x	x			(x)	(x)			[x]	[x]
<i>Pseudomonas aeruginosa</i> , <i>Ps. sp.</i>		x		[x]			x				(x)			x	[x]	[x]
Other nonfermentative bacilli	x	x		[x]			x	x			(x)	(x)		x	[x]	[x]
Gram-positive cocci																
<i>Staphylococcus aureus</i>			x	[x]	x	x	[x]		[x]	x			x			[x]
<i>Streptococcus pyogenes</i> (Group A)						[x]			[x]				[x]			[x]
Group D streptococci, including enterococci	x		x	[x]		x	[x]						x		[x]	[x]
<i>Streptococcus pneumoniae</i>			[x]										x		[x]	[x]
Other streptococci (alpha, and so forth)	x		x	[x]		x			[x]				[x]			[x]
Miscellaneous groups													x			[x]
<i>Neisseria meningitidis</i>	x		x	[x]												[x]
<i>Haemophilus influenzae</i>	x		x	[x]									x		[x]	[x]
Other facultative organisms																

*From the Section of Microbiology and the Infectious Disease Research Laboratory, Wilmington, Medical Center, Wilmington, Del. Abbreviations: AM, ampicillin; CB, carbenicillin; CF, cephalothin; CM, chloramphenicol; CC, clindamycin; EM, erythromycin; GM, gentamicin; KM, kanamycin; LN, lincomycin; DP, methicillin; NA, nalidixic acid; NF, nitrofurantoin; PN, penicillin G; PB, polymyxin B; SM, streptomycin; TE, tetracycline. x, Recommended for use with the species indicated; (x), recommended for use with urine isolates only; [x], recommended for use with blood culture isolates only.

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Table 34-4. Zone size interpretative chart, Kirby-Bauer method*

ANTIBIOTIC OR CHEMOTHERAPEUTIC AGENT	DISC POTENCY	INHIBITION ZONE DIAMETER TO NEAREST MM.		
		RESISTANT	INTERMEDIATE	SENSITIVE
Ampicillin ¹				
Enterobacteriaceae and enterococci	10 µg.	11 or less	12-13	14 or more
Staphylococci		20 or less	21-28	29 or more
Haemophilus		19 or less	—	20 or more
Bacitracin	10 U.	8 or less	9-12	13 or more
Carbenicillin	100 µg.			
Pseudomonas sp.		13 or less	14-16	17 or more
Proteus and Escherichia coli		17 or less	18-22	23 or more
Cephaloridine	30 µg.	14 or less	15-17	18 or more
Cephalexin	30 µg.	14 or less	15-17	18 or more
Chloramphenicol	30 µg.	12 or less	13-17	18 or more
Clindamycin ²	2 µg.	14 or less	15-16	17 or more
Colistin (polymyxin E) ³	10 µg.	8 or less	9-10	11 or more
Doxycycline	30 µg.	12 or less	13-15	16 or more
Erythromycin	15 µg.	13 or less	14-17	18 or more
Gentamicin	10 µg.	12 or less	—	13 or more
Kanamycin	30 µg.	13 or less	14-17	18 or more
Lincomycin	2 µg.	9 or less	10-14	15 or more
Methicillin ⁴	5 µg.	9 or less	10-13	14 or more
Nafcillin and oxacillin	1 µg.	10 or less	11-12	13 or more
Nalidixic acid ⁵	30 µg.	13 or less	14-18	19 or more
Neomycin	30 µg.	12 or less	13-16	17 or more
Nitrofurantoin ⁶	300 µg.	14 or less	15-16	17 or more
Penicillin G				
Staphylococci	10 U.	20 or less	21-28	29 or more
Other organisms ⁶	10 U.	11 or less	12-21 ⁷	22 or more
Polymyxin B ⁸	300 U.	8 or less	9-11	12 or more
Rifampin (when testing <i>Neisseria meningitis</i> susceptibility only)	5 µg.	24 or less	—	25 or more
Streptomycin	10 µg.	11 or less	12-14	15 or more
Sulfonamides ^{9,10}	300 µg.	12 or less	13-16	17 or more
Tetracycline ¹¹	30 µg.	14 or less	15-18	19 or more
Trimethoprim/sulfamethoxazole	25 µg.	10 or less	11-15	16 or more
Vancomycin	30 µg.	9 or less	10-11	12 or more

*Courtesy Alfred W. Bauer, Group Medical Center, Seattle, Wash., and John C. Sherris and W. Lawrence Drew, University Hospital, Seattle, Wash. See also reference 3 at end of chapter. Updated and modified by other investigators. See references 9 and 14.

¹The ampicillin disc is used for testing susceptibility to both ampicillin and hetacillin.

²The clindamycin disc is used for testing susceptibility to both clindamycin and lincomycin.

³The polymyxins diffuse poorly in agar, and the accuracy of the diffusion method is less than with other antibiotics. Resistance is always significant, but some relatively resistant strains of *Klebsiella* and *Enterobacter* may give zones in the lower end of the sensitive range (up to 15 mm.). When treatment of systemic infections due to susceptible strains is considered, it is wise to confirm the results of a diffusion test with a dilution method.

⁴The methicillin disc is used for testing susceptibility to all penicillin-resistant penicillins: methicillin, cloxacillin, dicloxacillin, oxacillin, and nafcillin. Methicillin-resistant strains of *Staphylococcus aureus* are best detected at 30°C.

⁵Urinary tract infections only.

⁶This category includes some organisms, such as enterococci and gram-negative bacilli, that may cause systemic infections treatable by high doses of penicillin G.

⁷Any of the commercially available 300 or 250 µg. sulfonamide discs can be used with the same standards of zone interpretation.

⁸The tetracycline disc is used for testing susceptibility to all the tetracyclines: chlortetracycline, demeclocycline, doxycycline, methacycline, oxytetracycline, rolitetracycline, minocycline, and tetracycline.

Haemophilus influenzae

Other facultative organisms

From the Section of Microbiology and the Infectious Disease Research Laboratory, Wilmington, Medical Center, Wilmington, Del. Abbreviations: AM, ampicillin; CB, carbenicillin; CF, cephalothin; CM, chloramphenicol; CC, clindamycin; EM, erythromycin; GM, gentamicin; KM, kanamycin; LN, lincomycin; DP, methicillin; NA, nalidixic acid; NF, nitrofurantoin; PN, penicillin G; PB, polymyxin B; SM, streptomycin; TE, tetracycline. x, Recommended for use with the species indicated; (x), recommended for use with urine isolates only; [x], recommended for use with blood culture isolates only.

zone may actually be a different bacterial species (a mixed, rather than a pure, culture) and should be subcultured, reidentified, and retested.

Interpretation of zone sizes

The diameters of the inhibition zones are then interpreted by referring to Table 34-4, which represents the NCCLS subcommittee's present recommendations.

The term "susceptible" implies that an infection caused by the strain tested may be expected to respond favorably to the indicated antimicrobial for that type of infection and pathogen. "Resistant" strains, on the other hand, are not inhibited completely by therapeutic concentrations. "Intermediate" implies that the isolant may respond to unusually high concentrations of the agent, due either to high dosage levels or in areas, such as the urinary tract, where the drug is concentrated. In other circumstances, intermediate results might warrant further testing if alternative agents are not available.

Limitations of the test

This modified Bauer-Kirby procedure has been standardized for testing rapidly growing isolants, particularly members of the Enterobacteriaceae, *Staph. aureus*, and *Pseudomonas* species; limited experience also suggests that the interpretative standards hold for *Haemophilus* and streptococci, if blood ("chocolatized" if required) is added to the Mueller-Hinton agar. *Streptococcus pyogenes* and *S. pneumoniae* are generally susceptible to penicillin G and are not routinely tested; however, in those patients hypersensitive to penicillin, the isolant may be tested against erythromycin or lincomycin.

In general, fastidious organisms requiring an increased CO₂ tension or an anaerobic atmosphere, or whose growth rate is unusually slow, do not lend themselves to susceptibility testing by the standardized disc-agar diffusion method; agar plate or broth dilution test procedures are recommended. Likewise, testing of *Neisseria*

gonorrhoeae by the described procedure is not recommended. Because of the interest recently generated by early reports on reliable susceptibility testing of anaerobes using the disc procedure, a subsequent section will consider this technique.

Quality control procedures

It is essential that some form of quality control be carried out in performing the disc procedure, to ensure precision and accuracy of the test results. The NCCLS subcommittee recommends that the tests be monitored daily with stock cultures of the Seattle strains of *Staph. aureus* (American Type Culture Collection 25923) and *Escherichia coli* (ATCC 25922), using antibiotic discs representative of those to be used in the testing of clinical isolants.¹⁴ These cultures may be grown on soy-casein digest agar slants and stored under refrigeration (4° to 8° C.), and should be subcultured to fresh slants every 2 weeks.

For testing, the cultures are inoculated to soy-casein digest broth tubes, which are incubated overnight and streaked to agar plates to obtain isolated colonies; these are then picked to broth and tested as described in the preceding sections.

The control strains may be used as long as there is no significant change in the mean inhibition zone diameters not otherwise attributable to technical error. If such changes occur, fresh strains should be obtained from a reference laboratory or other reliable source. Individual values of zone diameters and their permissible differences are indicated in Table 34-5.

Table 34-5 represents a more precise computation, based on standard statistical methods, than previous publications. It is described in NCCLS's *Revised Tentative Standards* (May 1973), free copies of which may be obtained from the Committee.*

Recently, it has been recommended that a well-characterized and confirmed strain

*National Committee for Clinical Laboratory Standards, Los Angeles, Calif.

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Table 34-5. Maximum acceptable standard deviations and mean zone diameters that should be expected with the *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923)*

ANTIMICROBIC	DISC CONTENT	MAXIMUM ACCEPTABLE STAND. DEV.	CURRENTLY ACCEPTED TRUE MEAN ZONE DIAMETER (MM)	
			<i>E. COLI</i>	<i>S. AUREUS</i>
Penicillin	10 units	2.9	†	31.5
Ampicillin	10 µg.			
Staphylococci		2.9	†	29.5
Enteric bacilli and enterococci		1.3	17.5	†
Methicillin	5 µg.	1.6	†	19.5
Nafcillin				
and oxacillin	1 µg.	1.3	†	—
Cephalothin	30 µg.	1.3	20.5	31.0
Cephaloridine	30 µg.	1.6	—	†
Carbenicillin	50 µg.			
<i>Pseudomonas</i> sp.		1.3	—	†
<i>Proteus</i> and <i>E. coli</i>		1.6	—	†
Chloramphenicol	30 µg.	1.9	24.0	22.5
Tetracycline	30 µg.	1.6	21.5	23.5
Erythromycin	15 µg.	1.6	11.0	26.0
Lincomycin	2 µg.	1.9	†	—
Clindamycin	2 µg.	1.6	†	—
Kanamycin	30 µg.	1.6	21.0	22.5
Neomycin	30 µg.	1.6	20.0	22.0
Streptomycin	10 µg.	1.3	16.0	18.0
Gentamicin	10 µg.	1.3	22.5	23.0
Sulfonamides	300 µg.	1.6	—	†
Nitrofurantoin	300 µg.	1.6	—	†
Nalidixic acid	30 µg.	1.9	—	†
Polymyxin B	300 units	1.3	14.0	†
Vancomycin	30 µg.	1.3	†	17.0

*NCCLS Subcommittee on Antimicrobial Susceptibility Testing: Performance standards for antimicrobial disc susceptibility tests as used in clinical laboratories, revised tentative standards, May 1973. In Balows, A., editor: Current techniques for antibiotic susceptibility testing, Springfield, Ill., 1974, Charles C Thomas, Publisher.

†Data not relevant; — data not yet established.

of *Pseudomonas aeruginosa* be added to the quality control system.¹¹ Apparently, some lots of Mueller-Hinton agar may contain increased concentrations of Ca⁺⁺ and Mg⁺⁺. Since *Staph. aureus* and *E. coli* are not effected by these ions, they will demonstrate no changes in zone sizes, but growth of *Ps. aeruginosa* is enhanced and will therefore demonstrate smaller zones. Thus, the effect of increased concentrations of these cations would influence the interpretation of susceptibility and should be predetermined.

Susceptibility testing of anaerobes

With the diverse spectrum of activity of various antimicrobial agents against clinically significant anaerobes, it is clearly apparent that a simple, rapid, reliable test for their susceptibility is in demand. Attempts to adapt the Bauer-Kirby disc-agar diffusion technique for predicting sensitivity of anaerobes has been found to be generally unsatisfactory,¹⁹ but a number of workers in the field of anaerobic bacteriology are attempting to correlate the zone diameters obtained by the disc test, with the MIC's

obtained by either broth or agar dilution techniques, with varying degrees of success. Chief among these has been the work of Sutter and colleagues,¹⁸ who have obtained a statistically good correlation with most of the antibiotics tested against a variety of known strains of anaerobes. The authors point out, however, that if predictions of the antibiotic sensitivity of **unidentified** isolants are to be made by the disc-diffusion procedure, it may be necessary to establish separate criteria for organisms that have different growth rates—slow, moderately rapid, or rapid—since this is one of the major variables that affect zone sizes.

Therefore, we feel that until a standardized, reproducible, and clinically correlated disc-diffusion technique for predicting antibiotic susceptibility of significant anaerobes becomes available, the reader is best directed to the employment of methodologies utilizing broth or agar dilution procedures.^{13,14}

As a guide to the microbiologist and clinician, Table 34-6 from a recent publication by Finegold and co-workers* is presented. This is based on their correlation of in vitro laboratory findings with an evaluation of clinical effectiveness.

Other uses for antibiotic discs

One unexpected advantage of performing disc-sensitivity tests on primary plates inoculated with clinical specimens likely to contain more than a single pathogenic species (such as sputum, throat swabs, or urine) is the likelihood of **uncovering** organisms overgrown by other bacterial species. For example, an agar plate inoculated with a mixed culture of staphylococci and streptococci and "disced" with two different antibiotics may result in two distinct patterns of inhibition zones. One zone may show an inhibition of the staphylococci, whereas colonies of the streptococci may be growing within that zone.

*In Kagan, B. M., editor: Antimicrobial therapy, ed. 2, Philadelphia, 1973, W. B. Saunders Co.

Table 34-6. Susceptibility of anaerobes to antimicrobial agents*

ANTIMICROBIAL AGENT	MICROAEROPHILIC AND ANAEROBIC COCCI	BACTEROIDES FRAGILIS	BACTEROIDES MELANINOGENICUS	FUSOBACTERIUM VARII	OTHER FUSOBACTERIUM SPECIES	EUBACTERIUM AND ACTINOMYCES	CLOSTRIDIUM PERFRINGENS	OTHER CLOSTRIDIA
Penicillin G	++++	+	++++	+++†	++++	++++	++++	++ to +++
Lincomycin	+++	++	+++	++	+++	++ to +++	++ to +++	+
Clindamycin	+++	+++	+++	+++	+++	+++	+++	++
Metronidazole	++	+++	+++	+++	+++	?	+++	? +++
Chloramphenicol	+++	+++	+++	+++	+++	+++	+++	+++
Tetracycline	++	++	+++	++	++ to +++	++	++	++
Erythromycin	++ to +++	+	++	+	+	++ to +++	+	++ to +++
Vancomycin	++ to +++	+	+	+	+	+++	+++	+++

*Chart courtesy of Sydney M. Finegold. From Kagan, B. M., editor: Antimicrobial therapy, ed. 2, Philadelphia, 1973, W. B. Saunders Co. ++++, Drug of choice; ++, good activity; +, moderate activity; -, poor or inconsistent activity.

†A few strains are resistant.

‡Rare strains resistant.

§Based on old studies (resistance may have developed subsequently).

[illegible]

Around the other disc there may be only colonies of staphylococci, the streptococci having been inhibited.

Since antibiotic discs demonstrate distinctly different inhibitory capacities, they may be used for the sole purpose of selectively isolating various microorganisms. Following Vera's suggestions,²⁰ it has been our practice to place discs containing penicillin (10 units), neomycin (30 μ g.), and bacitracin (10 units) on all primary plates inoculated with a potentially mixed flora. The inhibition zones around the neomycin discs have been particularly useful in exposing colonies of Group A beta hemolytic streptococci, pneumococci, enterococci, and other streptococci. *Haemophilus influenzae* has been isolated with ease from within the zones surrounding the 10-unit bacitracin disc on blood agar plates inoculated with sputum, and from material from the throat and nasopharynx. Penicillin discs (10 units) have aided in unmasking colonies of coliform bacilli, pseudomonads and species of *Proteus*, *Candida albicans*, and others. A 10-unit penicillin disc also is useful in revealing colonies of *Bordetella pertussis* on Bordet-Gengou plates of nasopharyngeal cultures. Kanamycin discs (30 μ g.) have been reported to be helpful in separating *Bacteroides* and *Clostridium* species from other wound bacteria when the plates are incubated anaerobically.²⁰

DETERMINATION OF ANTIBACTERIAL LEVEL OF SERUM DURING ANTIBIOTIC THERAPY

A direct method for determining the antibacterial potency of serum was first described by Schlichter and associates.^{15,16} We have evaluated the Schlichter method in many cases of acute infections (particularly subacute bacterial endocarditis, staphylococcal septicemia, enterococcal endocarditis, and others) and can recommend it as a valuable and practical guide to the antibiotic therapy of severe or complicated bacterial infections.

Technique of Schlichter test*

1. Subculture a recent isolate of the organism to infusion agar or blood agar slants and store in the refrigerator until the test is run, then subculture to a tube of broth early on the day of the test.
2. Obtain the first blood specimen before therapy, if possible; this serves as a control. Allow a 24-hour period to elapse after initiation of therapy in order to permit stabilization of absorption and excretion rates. Then take blood samples at any desired interval, at the low point of the blood concentration curve, if the patient is on intermittent dosage. Collect 10 ml. of the patient's blood in a sterile tube. On receipt in the laboratory, the clot is separated and the serum is obtained by centrifugation. The serum is then transferred to a sterile, rubber-stoppered tube; it may be titrated at that time (or within 2 to 3 hours if refrigerated) or frozen immediately in a slanting position and stored at $-20^{\circ}\text{C}.$, a temperature at which it remains stable for several days.
3. Prepare serial twofold dilutions of serum in 1 ml. amounts in Mueller-Hinton broth,[†] using eight sterile, gauze-stoppered Kahn tubes (use a separate pipet for each dilution). The first tube contains only undiluted serum, and a ninth tube contains only broth and serves as a culture control. The serum dilutions range from undiluted through 1:128. Very sensitive organisms, such as alpha hemolytic streptococci, may require dilutions up to 1:2,048.
4. To each tube of the series add 0.05 ml. of a 1:1,000 dilution of a 6-hour broth culture of the organism isolat-

*Modified by John A. Washington II, Head, Section of Clinical Microbiology, Mayo Clinic.²¹

†Use soy-casein digest broth or others, such as brain-heart infusion or Levinthal's broth, for organisms not growing in Mueller-Hinton medium.

ed from the patient. Also prepare a pour plate using 1 ml. of the inoculum.

5. Incubate the test at 36° C. for 18 to 24 hours and examine. The bacteriostatic end point is taken as the highest dilution in which no visible growth occurs. Because of the inherent turbidity of some sera, it is recommended that subcultures be made from each tube to a sector of a blood agar plate. To determine bactericidal end points, transfer 0.05 ml. from each tube showing no growth to a tube of thioglycollate medium. Mix and incubate at 36° C. for 72 hours. The tube showing no growth in thioglycollate is taken as the end point. Good growth should be evident in the control tube.
6. In cases where the organism grows slowly, a loopful of an overnight broth culture may be used as the inoculum. When microaerophilic or anaerobic bacteria have been isolated, the tubes should be incubated anaerobically.
7. Since thioglycollate medium contains sufficient agar to permit the growth of discrete colonies, one can determine by inspection the number of colonies growing out, and thus the degree of killing. It is thus recommended that one report the results as follows:
 - a. Complete killing at serum dilution____, with no growth in subculture.
 - b. Partial inhibition at serum dilution____, with 1 to 2+ growth in subculture.
 - c. Record and report the presence of less than 10 colonies at the serum dilution observed.
8. If the percentage of bactericidal activity is required, the following may be carried out:
 - a. Pipette 0.5 ml. of each tube showing no gross turbidity into tubes containing 12 ml. of melt-

ed and cooled (45° to 50° C.) brain-heart infusion agar, mix, and make pour plates.

- b. Incubate for 72 hours at 36° C.
 - c. On the basis of the inoculum colony count (step 4), calculate the lowest titers of serum dilution showing 99.9% and 100% bactericidal activity, and report accordingly.
9. Schlichter indicated that optimal antibiotic dosage (either single or combined drugs) had been achieved when a bactericidal level of 1:2 (complete inhibition in the first two tubes) had been demonstrated; others, however, believe that a bactericidal level of 1:8 should be the minimum effective level in problem cases. Dosage is adjusted according to results of the test and is maintained for the duration of the infection.

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Evidence for Nosocomial Transmission of *Candida albicans* Obtained by Ca3 Fingerprinting

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The moderately repetitive sequence Ca3 was used to fingerprint *Candida albicans* isolates from 32 patients hospitalized for more than 3 days, 17 recent admissions or outpatients, and 8 recently readmitted patients and 10 commensal isolates from the community in Wellington, New Zealand, plus isolates from 21 hospitalized patients, 26 outpatients or recent admissions, 4 recently readmitted patients, and 10 healthy individuals in the community in Auckland, New Zealand. In Wellington, isolates from patients hospitalized in Wellington Hospital for more than 3 days were genetically significantly less diverse than were isolates from outpatients or recent admissions or isolates from healthy individuals in the community. In addition, two clusters of genetically similar strains were isolated from hospitalized patients significantly more often than from other individuals. These observations provide evidence (albeit indirectly) for nosocomial transmission of hospital-specific *C. albicans* strains. In contrast, no indication of hospital-specific transmission of *C. albicans* was found in Auckland Hospital. Since these results were obtained under conditions in which no candidiasis outbreak occurred in either hospital, they also suggest that Ca3 fingerprinting may be a useful tool in preventive nosocomial infection control programs, allowing assessment of the extent of *C. albicans* transmission occurring in a hospital.

Most humans carry the yeast *Candida albicans* as part of their commensal microflora, but in hosts predisposed to candidiasis it can act as a pathogen. In hospitals, greater success in the treatment of cancers and in the treatment of viral and bacterial infections and improvement in intensive care have led to an ever-increasing number of severely compromised patients susceptible to candidiasis. This has led to an increase in the incidence of nosocomial candidiasis, with *C. albicans* being the most frequently encountered species. In most patients, candidiasis is localized, causing patients a considerable degree of discomfort. For certain high-risk groups of patients, such as burn patients, low-birth-weight babies, and leukemics, a 5 to 15% incidence of *Candida* fungemia with an associated mortality rate of up to 90% has been reported (1, 4, 6, 7, 22).

It is a commonly expressed opinion that strains which were already present as part of a host's commensal microflora are usually the etiological agents of candidiasis (7, 16, 20). On the other hand, clusters of nosocomial candidiasis do occur, suggesting the possibility of transmission of virulent strains to patients (5). It is important to determine whether transmission contributes to nosocomial candidiasis. If it does, prevention of transmission may reduce the incidence of the disease.

The numerous attempts to demonstrate nosocomial transmission of *C. albicans* have been recently reviewed by Hunter (5). Some of these studies have indeed found the same type of strain on multiple patients but suffered from problems regarding the discriminatory power and reproducibility of the typing methods used. These problems are exemplified by the results of multiple studies of the so-called "London outbreak." As

summarized by Hunter, repeat analyses of this outbreak yielded contradictory results not only when different typing methods were used but also when the same method (restriction fragment length polymorphism on ethidium bromide-stained gels of whole DNA digests) was used twice for the same isolates (5). In his 1991 review, Hunter concluded that nosocomial transmission of *C. albicans* had not yet been unequivocally demonstrated (5). Our own review of the literature indicates that to date this conclusion remains valid.

The present study employed computer-assisted Ca3 DNA fingerprinting (15), the most highly discriminating and accurate *C. albicans* typing method currently available (5, 8), to test if strain transmission between hospitalized patients occurs. Use was made of the ability of the method to quantitatively describe the relationships between groups of isolates on the basis of their genetic similarity (15). By this approach, nosocomial transmission should be revealed by the widespread occurrence of groups of genetically similar strains on hospitalized patients. These groups of strains should in addition be significantly less frequent on outpatients or recent admissions and healthy individuals in the community.

Using this approach, we have obtained evidence for transmission of *C. albicans* in one of two hospitals studied.

MATERIALS AND METHODS

Selection and isolation of strains. Clinical isolates were from patients at Wellington Hospital, Wellington, New Zealand, admitted to 16 different wards and from patients at Auckland Hospital, Auckland, New Zealand, admitted to 27 different wards. Both are tertiary-care hospitals of approximately the same size (546 and 687 beds, respectively). Isolates (one per patient) for fingerprinting were chosen from isolates recovered from clinical specimens submitted to hospital microbiological laboratories between August 1992 and November 1993. All isolates had been identified as *C. albicans* by the germ tube assay (7). This identification was verified by the results of fingerprinting with the species-specific probe Ca3. All isolates had the strong and complex banding patterns characteristic of *C. albicans* but absent in related species (9, 13, 18, 19). All isolates

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fingerprinted were from sites at which yeast concentrations exceeded the levels for commensal colonization listed by Odds (7). Yeast concentrations were determined by semiquantitative plate-streaking procedures employed by the laboratories. These results were converted to cell numbers by using conversion factors derived from calibration experiments with samples of known viable-cell concentrations. Vaginal isolates were excluded from the analysis because earlier studies have indicated that relationships among these isolates differ from those among isolates found in other body locations and that they must thus be assessed separately (11, 14). Commensal isolates (each from a different person) were obtained from nonrelated healthy volunteers in the community. For each isolate, a single colony was used to inoculate an agar slant which formed the basis of further analysis. Additional information on the histories of the isolates is summarized in Table 1 and in Fig. 1 and 5.

DNA fingerprinting with the Ca3 probe. DNA fingerprinting was performed as previously described by Schmid et al. (15). In brief, cells were grown in YPD medium to late log or early stationary phase and DNA was prepared from spheroplasts according to the method of Scherer and Stevens (10). The DNA concentration was determined fluorometrically by using the dye H 33258 (3). DNA was digested with *EcoRI*, after which fragments were separated on 0.8% agarose gels and transferred to a nitrocellulose membrane and then subjected to Southern hybridization with a ³²P-labelled nick-translated Ca3 probe (15) and autoradiography. The Ca3 probe contains 11 kb of repetitive sequences and hybridizes to all but two chromosomes of *C. albicans* (2). For a detailed description of its characteristics as a fingerprinting tool, see reference 15.

Analysis of fingerprints for the determination of relationships between strains. The methods used for analysis of fingerprints were those described by Schmid et al. (15). Southern blot patterns were entered into a Macintosh Ilii computer as data files. The Dendron computer program (15) was used to quantify the degree of similarity between patterns of different isolates, resulting in similarity values, also referred to as S_{AB} values, ranging from 1.0 (the two patterns compared are identical) to 0.0 (the two patterns have no bands in common) (15). Relationships between isolates were visualized in dendrograms constructed from matrices of similarity values by using the unweighted pair group method (17). The branching point between two isolates in dendrograms reflects their genetic similarity. Groups of genetically similar strains were defined as groups of isolates connected by branching points in dendrograms at an S_{AB} value of ≥ 0.8 ; this value lies halfway between the average S_{AB} value found for multiple isolates from the same patients (0.96) (reference 13 and this study) and the average S_{AB} value for isolates from different unrelated healthy individuals in the community (0.66) (references 13, 15, and 18 and this study).

Test for the preferred association of groups of genetically similar strains with patients with particular characteristics. The frequency of isolation of a group of genetically similar strains (defined as described above) from patients showing a particular characteristic (e.g., a particular sex or age, long hospitalization, or admission to a particular ward) was determined. Next, the frequency of the same group of strains among other patients lacking this characteristic (or having the opposite characteristic) was determined. A z test was used to determine if the difference in frequency was statistically significant.

RESULTS

Analysis of Wellington isolates. Sixty-seven *C. albicans* isolates, each from a different individual, were analyzed (Fig. 1). These included 10 commensal isolates (labelled C in Fig. 1) and 57 patient isolates. Of the 57 patients, 32 had been hospitalized for more than 3 days prior to the isolation of *C. albicans* and were categorized by us for the purpose of this study as hospitalized (labelled H in Fig. 1). Seventeen were either outpatients or had been hospitalized for 3 days or less and were categorized by us as outpatients or recent admissions (labelled OR in Fig. 1). Eight additional patients were recent admissions but had been hospitalized within the 6 preceding months and were for that reason not included in the outpatient-or-recent-admission category).

Figure 1 shows that many of the strains were genetically dissimilar. However, several groups of similar isolates are also apparent (such groups being defined as containing isolates with S_{AB} values of ≥ 0.8 between them). The three largest groups of genetically similar isolates are labelled WA, WB, and WC in Fig. 1. Several of the isolates within these groups were as, or almost as, similar to each other as were multiple isolates from the same patients (average S_{AB} value = 0.96 ± 0.02 , determined with two isolates each from four patients). Figure 2A gives an overview of the range of Ca3 patterns encountered; Fig. 2B illustrates the similarities between isolates from the

TABLE 1. Histories of *C. albicans* isolates used in this study

Isolate or patient category	No. (%) of isolates	
	Wellington	Auckland
Patient isolates		
No. of isolates	57 (100)	51 (100)
Sex of patients		
Female	23 (40)	21 (41)
Male	34 (60)	30 (59)
Age of patients (yr)		
0-2	3 (5)	4 (8)
2-50	17 (30)	16 (31)
>50	37 (65)	31 (61)
Most frequent sites of <i>C. albicans</i> isolation		
Sputum or aspirate	16 (28)	18 (35)
Skin or wounds	14 (24)	11 (22)
Feces	11 (19)	0 (0)
Urine	0 (0)	9 (18)
Oral cavity	10 (17)	7 (14)
Most frequent conditions predisposing patient to candidiasis ^a		
Antibacterial antibiotics	13 (23)	12 (24)
Surgery	11 (20)	5 (10)
Catheterization	4 (7)	6 (12)
Asthma (corticosteroid treatment)	10 (17)	1 (2)
Diabetes	0 (0)	3 (6)
No. of patients at high risk of acquiring candidiasis ^b	9 (15)	8 (16)
No. of patients with clinical features of candidiasis reported	32 (56)	17 (33)
Duration of hospitalization of patient prior to isolation of <i>C. albicans</i> (days)		
>3 ^c	32 (56)	21 (41)
0-3 ^d	17 (30)	26 (51)
0-3 readmission ^e	8 (14)	4 (8)
Commensal isolates		
No. of isolates	10	10
Sex of patients		
Female	6	4
Male	4	3
Unknown	0	3
Age of patients (yr)		
0-2	0	0
2-50	9	6
>50	1	1
Unknown	0	3
Site of <i>C. albicans</i> isolation	Oral cavity	Oral cavity

^a Conditions listed by Odds (7) as predisposing patients to candidiasis.

^b HIV-positive or leukemia patients or those undergoing anticancer therapy.

^c The average durations of hospitalization were 32 ± 65 days in Wellington Hospital and 29 ± 26 days in Auckland Hospital.

^d Outpatients and recent admissions. The average duration of hospitalization was 1 ± 1 day in both hospitals.

^e Recent admissions who had been hospitalized within the previous 6 months.

same patient and isolates from different patients falling into the same group of genetic similarity.

If transmission of *C. albicans* between hospitalized patients occurs, isolates from such patients should be derived from a limited number of groups or clusters whose members are sim-

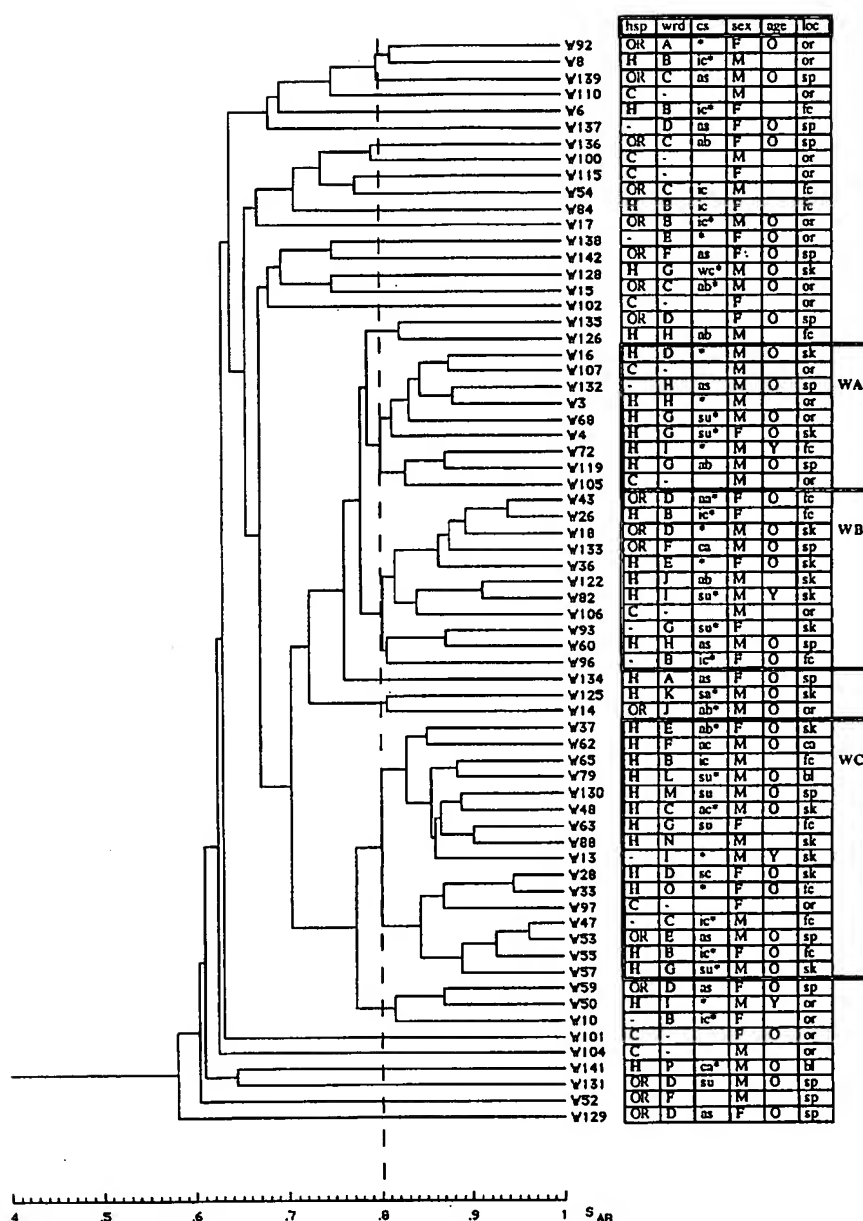


FIG. 1. Genetic relationships among all Wellington isolates determined on the basis of the similarity of their Ca3 fingerprints. The dashed line in the dendrogram denotes the threshold S_{AB} value of 0.8 for defining groups of genetic similarity. The three largest groups of genetically similar isolates, WA, WB, and WC, are marked on the right of the dendrogram. Following the designation of each isolate is information on the duration of hospitalization of the patient (column labelled hsp; H, hospitalized for >3 days; OR, outpatient or recent admission; -, recent admission who was hospitalized previously; C, individual in the community [commensal isolate]), the wards to which patients had been admitted (column labelled wrd), clinical status indicating conditions predisposing the patient to candidiasis (column labelled cs; aa, asthma plus antibiotics; ab, antibiotics; ac, antibiotics plus catheterization; as, asthma; ca, catheterization; ic, immunocompromised by HIV, leukemia, or cancer or cancer treatments; sa, surgery plus antibiotics; su, surgery; wc, wound plus catheter). Asterisks denote patients for whom clinical symptoms indicating candidiasis were reported, sex, age (O, >50 years; Y, <2 years; blank cells, 2 to 50 years), and the body site or material from which the isolate was obtained (column labelled loc; bl, bloodstream; ca, catheter; fc, feces; or, oral cavity; sk, skin, including wounds; sp, sputum).

ilar to each other. In contrast, isolates from recently admitted patients, outpatients, or healthy individuals in the community should be genetically more diverse. Figure 1 indicates that many of the isolates from hospitalized (>3 days) patients in Wellington Hospital were derived from only a few groups, since a high percentage of them were associated with the three large clusters WA, WB, and WC.

To directly assess if a difference in genetic diversity existed

between isolates from hospitalized patients and isolates from outpatients or recent admissions or commensal isolates, we determined the relationships between isolates in each category separately (Fig. 3). Isolates from hospitalized patients were indeed genetically less diverse than were isolates from the other two categories: in dendrograms (Fig. 3A), more than 75% of isolates from hospitalized patients formed clusters with other similar isolates from such patients. In contrast, only 24%

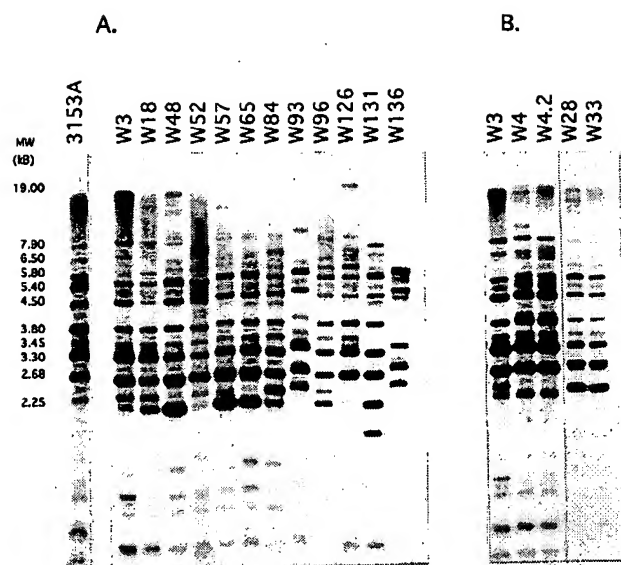


FIG. 2. (A) Range of DNA fingerprints of isolates from different individuals in Wellington plus strain 3153A, which is included as a standard, and (B) examples of fingerprints of isolates from the same groups of genetic similarity (W3 and W4 from group WA and W28 and W33 from group WC) in comparison with fingerprints of multiple isolates from the same patient (W4 and W4.2, isolated on the same day from a wound and a drain site, respectively). Molecular sizes (MW) shown are those of the bands of standard strain 3153A.

of isolates from outpatients or recent admissions and only 20% of isolates from healthy individuals in the community formed clusters. Conversely, the frequency of S_{AB} values of ≥ 0.8 (the threshold defining groups of similarity) was 21% among isolates from patients hospitalized for >3 days, compared with only 7% among isolates from outpatients or recent admissions and 2% among commensal isolates from healthy individuals (Fig. 3B). The differences in the frequency of high S_{AB} values between isolates from hospitalized patients and isolates either from outpatients or recent admissions or from healthy individuals were statistically significant ($P < 0.0010$ and $P < 0.0005$, respectively, by the z test).

We next sought to identify the group(s) of genetically similar strains that was being spread within the hospital. Because of their transmission in the hospital, these strains should occur more frequently in hospitalized patients than in individuals in the community or recent admissions. Figure 4 shows a comparison of the distributions of the three main groups of similar strains within populations of healthy individuals in the community, outpatients or recent admissions, and hospitalized patients. Members of one of the clusters, WB, were isolated with approximately the same frequency (15 to 17%) both outside and within the hospital environment. Members of the cluster WC were in contrast isolated from only 7% of individuals belonging to the group of outpatients or recent admissions plus healthy subjects but from 38% of hospitalized patients, and this difference was statistically significant (z test, $P < 0.005$). The differences in the frequency of isolation of strains from cluster WC between hospitalized patients and either outpatients or recent admissions (38 versus 6%) or healthy individuals in the community (38 versus 10%) were also statistically significant (z test, $P < 0.010$ and $P < 0.050$, respectively). The presence of strains of this cluster was not restricted to a limited number of wards. The 12 hospitalized (>3 days) patients from whom WC strains were isolated had been admitted to 10 different wards.

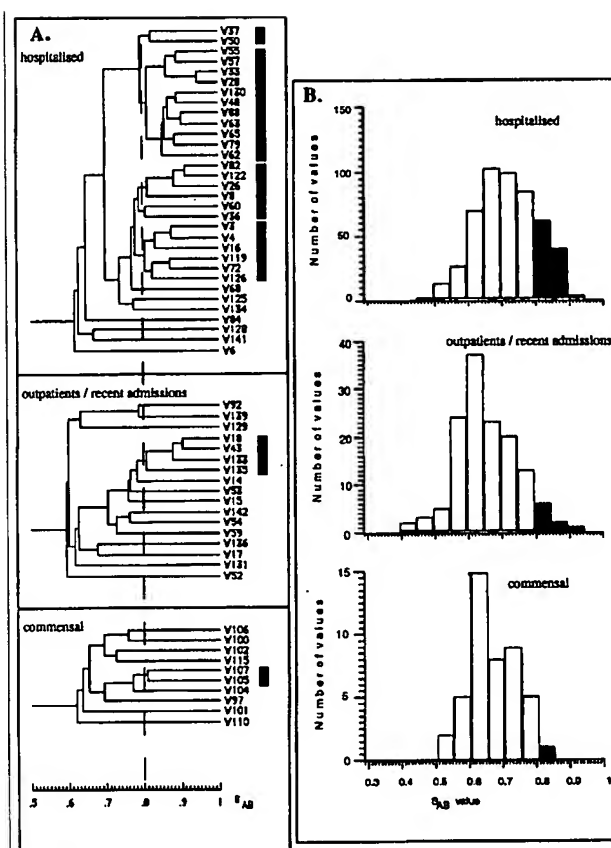


FIG. 3. Comparison of genetic diversity among groups of Wellington isolates from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals in the community (commensal). (A) Dendrograms of each of the three groups. The dashed lines mark the threshold defining groups of genetic similarity ($S_{AB} = 0.8$); the groups are marked by bars on the right of the dendrogram. (B) Histograms of the distribution of S_{AB} values among isolates from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals from the community. Shaded bars mark S_{AB} values of >0.8 .

WC thus represents a widespread hospital-specific group. Strains belonging to the cluster WA were somewhat more frequently isolated from hospitalized patients (frequency of isolation = 19%) than from individuals outside the hospital (frequency of isolation = 8%), but this difference was not statistically significant. When individual wards were analyzed, it became apparent that strains of this group were found more often on patients from each of two of the wards than on individuals from the remainder of the sample. WA strains were isolated from 3 of 7 (or 43%) ward G patients but from only 6 of 60 (or 10%) other individuals. WA strains were also isolated from 2 of 4 (or 50%) ward H patients but from only 7 of 63 (or 11%) other individuals. The number of patients in the two wards was small, but the differences in frequency were statistically significant (z test, $P < 0.050$ and $P < 0.025$, respectively). All of the ward G and ward H patients from whom the WA strains were isolated had been hospitalized for more than 3 days, with one exception: patient W132 had been admitted only 1 day before sampling but had been previously admitted to the same ward only 2 weeks earlier. WA strains may thus also be a group that is being transmitted within the hospital, though only within a limited number of wards.

We next sought to determine if isolates in any of the clusters WA, WB, and WC were more frequently isolated from any

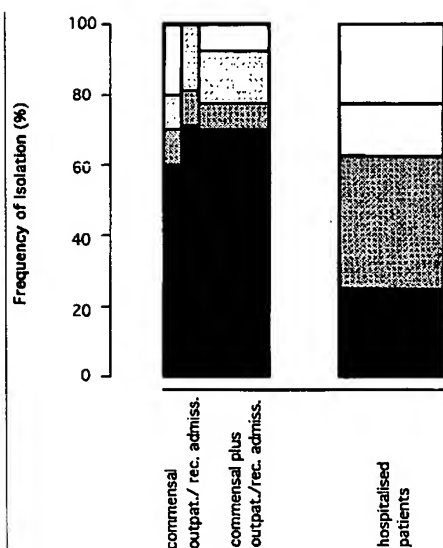


FIG. 4. Frequency of isolation of the major groups of genetically similar Wellington strains, WA (□), WB (▨), and WC (■), from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals in the community. ■, other strains.

subgroups of patients defined by age, gender, predisposing conditions, or body site of isolation, indicating adaptation of groups of genetically similar strains to patients with certain physiological or clinical features. We also tested whether any of the clusters were more frequently isolated from patients for whom clinical features of candidiasis had been reported. No statistically significant correlations were observed.

Analysis of Auckland isolates. Sixty-one *C. albicans* isolates, each from a different individual, were analyzed (Fig. 5). These included 10 commensal isolates (labelled C in Fig. 5) and 51 patient isolates. Of the 51 patients, 21 had been hospitalized for more than 3 days prior to the isolation of *C. albicans* (labelled H in Fig. 5). Twenty-six either were outpatients or had been hospitalized for 3 days or less (labelled OR in Fig. 5). Four additional patients were recent admissions but had been hospitalized within the 6 preceding months and were for that reason not included in the outpatient-or-recent-admission category.

Figure 5 gives an overview of the genetic relationships among the 61 strains. As in Wellington, many of the strains were genetically dissimilar but several groups of genetic similarity were also apparent. The two largest, containing 13 and 11 isolates, respectively, are labelled AA and AB in Fig. 5. Examples of the actual Ca3 patterns are shown in Fig. 6.

In contrast to the one resulting from our analysis of the Wellington isolates, the dendrogram in Fig. 5 does not suggest that isolates from hospitalized patients in Auckland were frequently derived from the main clusters of genetically similar strains. This impression was confirmed by the results of further tests which gave no indication of transmission in Auckland Hospital: an analysis of the genetic diversity of isolates from hospitalized patients, outpatients or recent admissions, and healthy individuals (Fig. 7) showed that isolates from hospitalized patients were not less diverse than isolates from outside the hospital environment were. In the dendrograms in Fig. 7A, 42% of the isolates from hospitalized patients formed clusters with other similar isolates compared with 69% of isolates from outpatients or recent admissions and 30% of commensal isolates. Conversely, the frequency of high S_{AB} values (≥ 0.8) was 9.5% among isolates from hospitalized patients, compared

with 18% among isolates from outpatients or recent admissions and 7% among isolates from healthy individuals in the community. There was also no significant difference in the frequency with which strains from the major clusters, AA and AB, were encountered within and outside the hospital environment (Fig. 8). Likewise, no association of either group of strains with patients on any specific ward(s) was found.

Hospital-specific transmission could have been masked in the above-described analysis by strong preferences of groups of genetically similar strains for particular types of patients (defined, for instance, by physiological or clinical condition) or body sites, regardless of hospitalization. We therefore tested whether either of the two large groups of genetically similar strains was more frequently encountered in subgroups of patients defined by age, gender, predisposing conditions, reported clinical symptoms of candidiasis, or body site than on the remainder of the patients. We found the following statistically significant (z test, $P < 0.05$ or less) correlations. Strains of group AA were encountered 3.4 times more often in patients at high risk of acquiring candidiasis (human immunodeficiency virus (HIV)-positive and cancer patients), and 2.9 times more often in the urinary tract than at other sites. Strains of group AB were encountered 6.4 times more often in patients above the age of 50 than in other patients. None of these prevalences were higher for hospitalized patients than for outpatients or recent admissions, which would have indicated transmission in the hospital between certain patient groups.

The prevalence of group AA strains in the urinary tract was actually highest outside the hospital environment, and the high-risk patients were almost exclusively outpatients or recent admissions. These two factors could thus have concealed a reduced genetic diversity among isolates from hospitalized patients. We therefore repeated the comparison of genetic diversity between isolates from hospitalized and nonhospitalized patients with a sample of isolates from which urinary tract isolates and isolates from high-risk patients had been omitted. The results (data not shown) provided neither evidence nor even a trend pointing towards either reduced genetic diversity among isolates from hospitalized patients or an increased frequency of the remaining cluster AA and cluster AB isolates in hospitalized patients—again providing no indication of hospital-associated transmission.

Whereas the above-described analyses yielded no indication of transmission in Auckland Hospital, they did highlight some differences between the strains isolated from patients and those isolated from healthy individuals. The level of genetic diversity of strains from outpatients or recent admissions was significantly lower (z test, $P < 0.025$) than was that of strains from healthy individuals (18 versus 7% of S_{AB} values of ≥ 0.8). In addition, strains from groups AA and AB were isolated from 14 and 24% of hospitalized (>3 days) patients, respectively, and from 34 and 23% of outpatients or recent admissions but were not isolated from any of the 10 healthy individuals. With the exception of the difference in frequency of isolation of AA strains between hospitalized patients and healthy individuals, all these differences were statistically significant (z test, $P < 0.050$ or less). The populations of strains found in both outpatients or recent admissions and hospitalized patients were thus not identical to the population of commensal strains in healthy individuals.

DISCUSSION

It was the aim of this study to assess whether *C. albicans* strains can be transmitted to patients within the hospital environment by using a retrospective comparison of isolates ob-

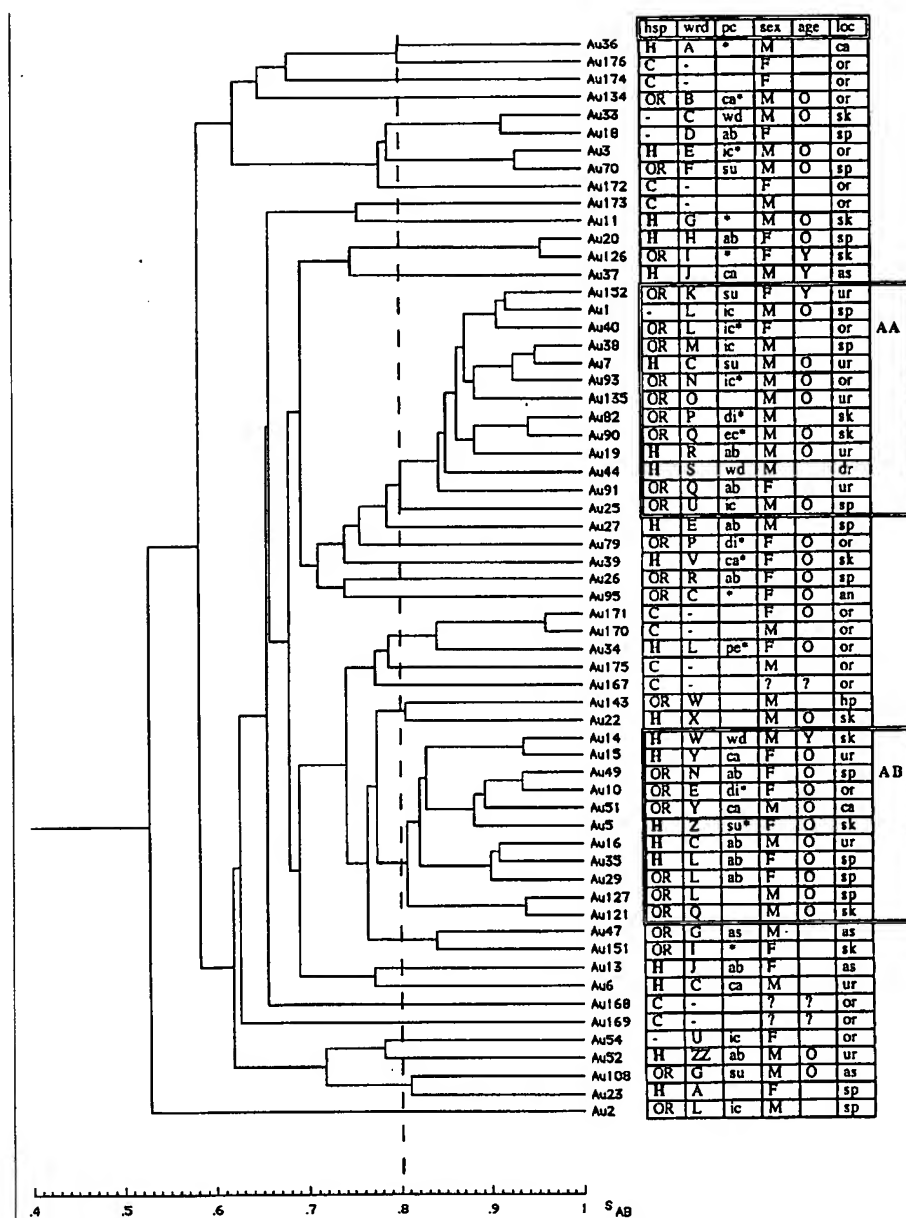


FIG. 5. Genetic relationships among all Auckland isolates determined on the basis of the similarity of their Ca3 fingerprints. The dashed line in the dendrogram denotes the threshold S_{AB} value of 0.8 for defining groups of genetic similarity. The two largest groups of genetically similar isolates, AA and AB, are marked on the right of the dendrogram. Following the name of each isolate is information on the duration of hospitalization of the patient (column labelled hsp; H, hospitalized for >3 days; OR, outpatient or recent admission; -, recent admission who was hospitalized previously; C, individual in the community [commensal isolate]), the wards to which patients had been admitted (column labelled wrd), the clinical status indicating conditions predisposing the patient to candidiasis (column labelled pc; ab, antibiotics; as, asthma; ca, catheterization; di, diabetes; ec, eczema; ic, immunocompromised by HIV, leukemia, or cancer or cancer treatments; pe; pleural effusion; su, surgery; wd, wound. Asterisks denote patients for whom clinical symptoms indicating candidiasis were reported), sex, age (O, >50 years; Y, <2 years; blank cells, 2 to 50 years), and body site or material from which the isolate was obtained (column labelled loc; an, anus; as, aspirate; ca, catheter; dr, drain; hp, hip; or, oral cavity; sk, skin, including wounds; sp, sputum; ur, urine).

tained from patients who had been hospitalized for different lengths of time. In one of two hospitals surveyed (Wellington Hospital), the majority of isolates obtained from patients after prolonged hospitalization was derived from a limited number of groups of genetically similar strains. Strains from outpatients or recent admissions and healthy individuals were, in contrast, significantly more dissimilar to each other, i.e., genetically more diverse. We identified two groups of genetically

similar strains which were significantly more prevalent in patients after prolonged hospitalization, one (WC) found in patients from a large number of different wards and one (WA) found in patients from two specific wards. No other patient characteristics, such as sex, age, or physiological or clinical state, were correlated with the increased prevalence of these clusters. These observations thus indicate that in Wellington Hospital, hospitalization led to the frequent replacement of

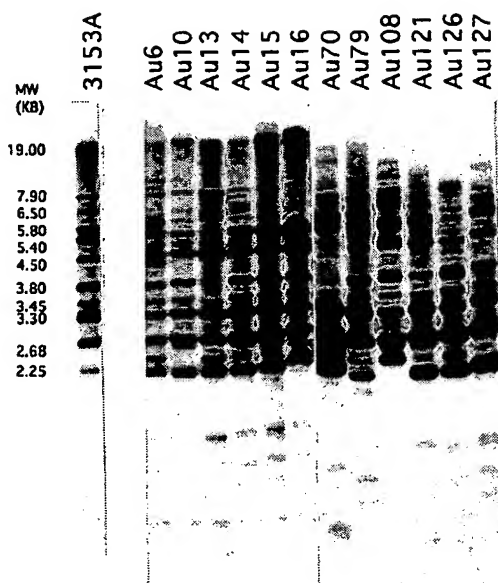


FIG. 6. Range of DNA fingerprints of isolates from different individuals in Auckland plus strain 3153A, which is included as a standard. Molecular sizes (MW) shown are those of the bands of standard strain 3153A.

patients' strains with strains acquired in the hospital environment and therefore provide evidence (albeit indirectly) for nosocomial transmission of *C. albicans*.

The fact that our evidence indicates transmission of groups of similar strains rather than of a single strain allows some conclusions to be drawn regarding the possible scenario underlying these transmissions. It seems likely that the progenitors of the two groups of strains transmitted became established in the hospital in the past and that their progeny have since evolved while spreading throughout the hospital environment; such spread may involve not only contact between human hosts but also inanimate surfaces and food (21). We are now in the process of identifying the reservoirs of these strains and vehicles of their transmission in the hospital environment. Our findings also suggest one reason why previous attempts to demonstrate nosocomial transmission of *C. albicans* have yielded inconclusive results (5). Since the methods employed lacked the capability to group strains according to their similarity, they could have detected only single-strain transmission and not transmission of groups of genetically similar strains.

In the second hospital studied (Auckland Hospital), we found no indications of nosocomial transmission of *C. albicans*. We emphasize that this does not imply that compromised patients in this hospital always retained their commensal strains. The frequency of the major groups of strains, AA and AB, in patients (both hospitalized patients and outpatients or recent admissions) was higher than their frequency in healthy individuals. In addition, the level of genetic diversity of patient isolates was lower than that of commensal isolates. These differences indicate that commensal strains had been replaced but that replacement had occurred regardless of hospitalization, implicating the community as the source of the replacing strains. This assumption is corroborated by the results of an earlier study in which we demonstrated strain replacement in nonhospitalized AIDS patients (13). The design of the present study did not allow us to determine when strain replacement in hospitalized patients took place. On the basis of our earlier work (13), it seems likely that replacement may have occurred

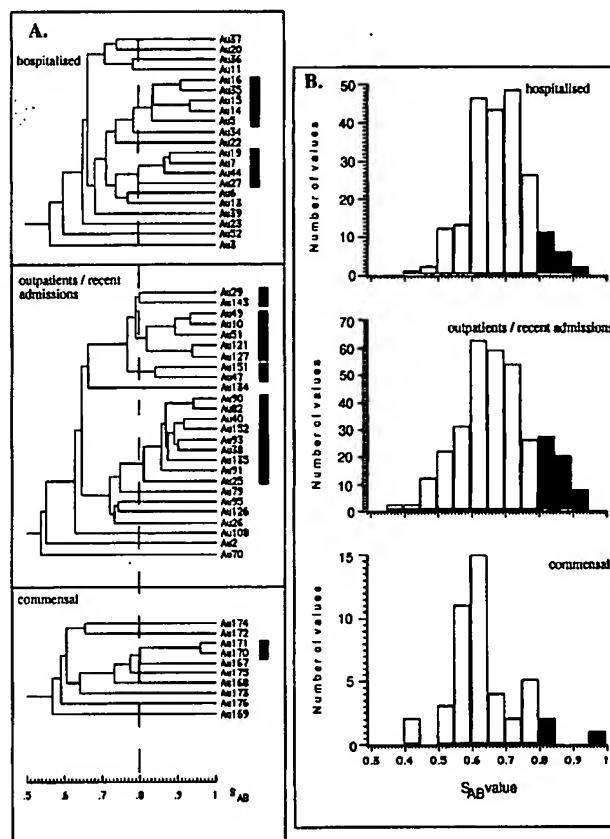


FIG. 7. Comparison of genetic diversity among groups of Auckland isolates from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals in the community (commensal). (A) Dendrograms for each of the three groups. The dashed lines mark the threshold defining groups of genetic similarity ($S_{AB} = 0.8$); the groups are marked by bars on the right of the dendrograms. (B) Histograms of the distribution of S_{AB} values among isolates from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals from the community. Shaded bars mark S_{AB} values of >0.8.

prior to the patients' hospitalization. Certain groups of replacing strains were found more frequently on patients with particular characteristics, regardless of whether the patients were hospitalized: group AA preferably associated with high-risk patients and patients with urinary tract disorders, and group AB preferably associated with patients older than 50 years. This, in turn, raises the possibility of specific adaptation of groups of strains towards colonization of certain types of patients. However, in a combined analysis of isolates from a number of New Zealand hospitals these strains grouped together with strains from other centers showing no such preferences (12). This makes it more likely that the preferred association of the Auckland groups with certain types of patients was based on routes of transmission existing within these patient populations.

The observation that nosocomial transmission of *C. albicans* apparently occurs only in some hospitals and not in others raises questions as to the reasons for these differences. One possible explanation would be that the groups of strains transmitted in Wellington Hospital were genetically well adapted to survival in the hospital environment and that strains with such capabilities were not present in Auckland. This appears unlikely because in a combined Ca3 analysis of isolates from several hospitals in New Zealand (12), we found that the

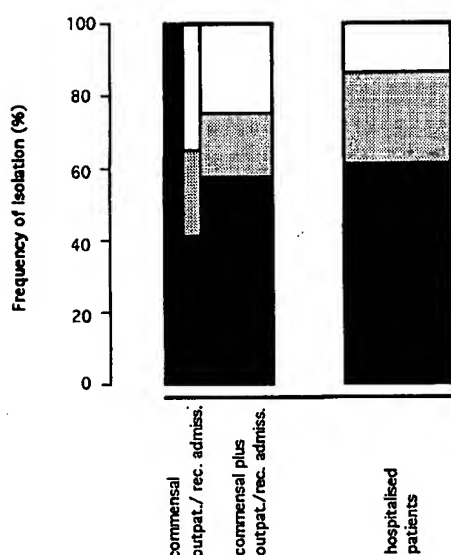


FIG. 8. Frequency of isolation of the major groups of genetically similar Auckland strains, AA (□) and AB (▨), from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals in the community (commensal). ■, other strains.

strains transmitted in Wellington (groups WA and WC) formed clusters of genetic similarity with Auckland strains belonging to groups AA and AB. Further studies will be required to assess which epidemiological factors may be responsible for the observed differences in the frequency of transmission between the two hospitals.

In addition to providing evidence for nosocomial *C. albicans* transmission, the results of this study suggest potential applications for Ca3 fingerprinting in preventing candidiasis outbreaks. Because of the high resolution of Ca3 fingerprinting, occasional spot testing of fairly small numbers of select isolates submitted to the clinical laboratory is apparently sufficient to determine whether transmission of *C. albicans* occurs in a given hospital, even in the absence of an obvious outbreak situation. Ca3 fingerprinting is thus a sensitive first screen for the detection of nosocomial transmission of *C. albicans*, and if the fingerprinting analyses were contracted out to laboratories in which the methodology is established, such screens would also be rapid and inexpensive. In instances in which such a spot test indicated transmission, the method could potentially be utilized to track reservoirs and routes of transmission by sampling of the hospital environment and caregivers. This may enable a hospital to eliminate such routes before they become the means of transmission of highly virulent strains causing single-strain outbreaks.

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Invasive aspergillosis presenting as pericarditis and cardiac tamponade

JM Luce, RC Ostenson, SC Springmeyer and LD Hudson

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A M E R I C A N C O L L E G E O F



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occurred in association with probable pneumoconiosis: Carasso et al³ reported a 48-year-old woman with a bronchoesophageal fistula due to silicotic mediastinal lymphadenopathy, and Frew⁴ reported a 51-year-old man with a chest roentgenogram suggestive of pneumoconiosis and dysphagia due to a tuberculoma in the esophagus. Mediastinal lymph node enlargement due to other granulomatous diseases such as sarcoidosis and histoplasmosis have been shown to compress the esophagus.¹⁻²

Although this patient had no specific signs or symptoms of tuberculosis, tissue cultures of the cervical lymph nodes grew *M intracellulare*. The increased incidence of *Mycobacterium tuberculosis* in silicotic patients is well known. Also atypical mycobacterial infections appear to be more frequent in patients with pneumoconioses.⁵⁻⁶ Blacks generally have a lower incidence of clinical atypical mycobacteriosis than whites, but our patient's silicosis and diabetes mellitus may have increased his risk to infection.⁵ His anemia of chronic disorders may be related to the mycobacteriosis.⁷

Although this patient was lost to follow up by the chest service, it is interesting to speculate what the most appropriate course of therapy for him would have been. We recommended that he undergo a thoracotomy to remove the mediastinal lymph nodes compressing the esophagus and perhaps reduce the burden of tuberculous tissue as well. We also would have administered chemotherapy with five to six antituberculosis drugs because *M intracellulare* is notoriously resistant to conventional therapy.⁸ Atypical mycobacterioses are even more difficult to treat in patients with pneumoconioses.⁶

This is the first reported case, to our knowledge, in which silicotic lymphadenopathy complicated by *M intracellulare* infection produced dysphagia by extrinsic compression of the esophagus. Silicosis should be considered among other granulomatous diseases such as mediastinal granuloma, tuberculosis, and sarcoidosis in the differential diagnosis of dysphagia.

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Invasive Aspergillosis Presenting as Pericarditis and Cardiac Tamponade*

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Leonard D. Hudson, M.D., F.C.C.P.

A 38-year-old leukemic patient developed pericarditis and cardiac tamponade due to *Aspergillus niger* one month after undergoing bone marrow transplantation. She failed to improve even though amphotericin B and rifampin therapy had been initiated before infection was evident. Her unique case illustrates both the unusual presentations of invasive aspergillosis and the difficulty of diagnosing and treating this increasingly common disease

Invasive aspergillosis is recognized with increased frequency among immunocompromised patients.¹⁻³ However, recognition often is delayed by the unusual and nonspecific manifestations of this condition. We present the case of a bone marrow transplant recipient in whom pericarditis and cardiac tamponade were the first clinical indications of invasive aspergillosis.

CASE REPORT

The patient was a 38-year-old woman with acute myelomonocytic leukemia who was transferred to the Fred Hutchinson Cancer Research Center (FHCRC; UPN907) in second relapse. Previous chemotherapy had included cytarabine, 6-thioguanine, and daunomycin. Admission laboratory findings included pancytopenia with a peripheral neutrophil count of 150/cu mm. Chest roentgenogram and ECG were normal (Fig 1, left). The patient was placed in laminar air flow and was given prophylactic oral antibiotics. She was prepared for transplantation with 1 mg/kg/body weight of nitrogen mustard followed by 1,200 rads of total body irradiation in six divided doses, as per FHCRC protocol.⁴

During preparation, the patient was given intravenous carbenicillin and gentamicin for fever. Intravenous amphotericin B, 25 mg/day, and rifampin, 300 mg/day, were added when blood cultures grew *Candida tropicalis*. Two weeks after admission, the patient received a bone marrow transplant from her HLA-matched sibling, who also was the source of daily granulocyte infusions.

The patient's posttransplantation course was complicated by an episode of pulmonary edema which was thought to be due to pre-existent anthracycline cardiac toxicity and fluid overload. Her respiratory status improved with diuretics, and her chest roentgenogram cleared but for a small right middle lobe (RML) infiltrate. She then developed toxic enteritis which was treated with intravenously administered corticosteroids.

Three weeks after admission, the patient noted anterior chest pain which radiated into her throat and was intensified in the supine position. The pain was felt to be due to *Candida* esophagitis after barium swallow disclosed esophagi-

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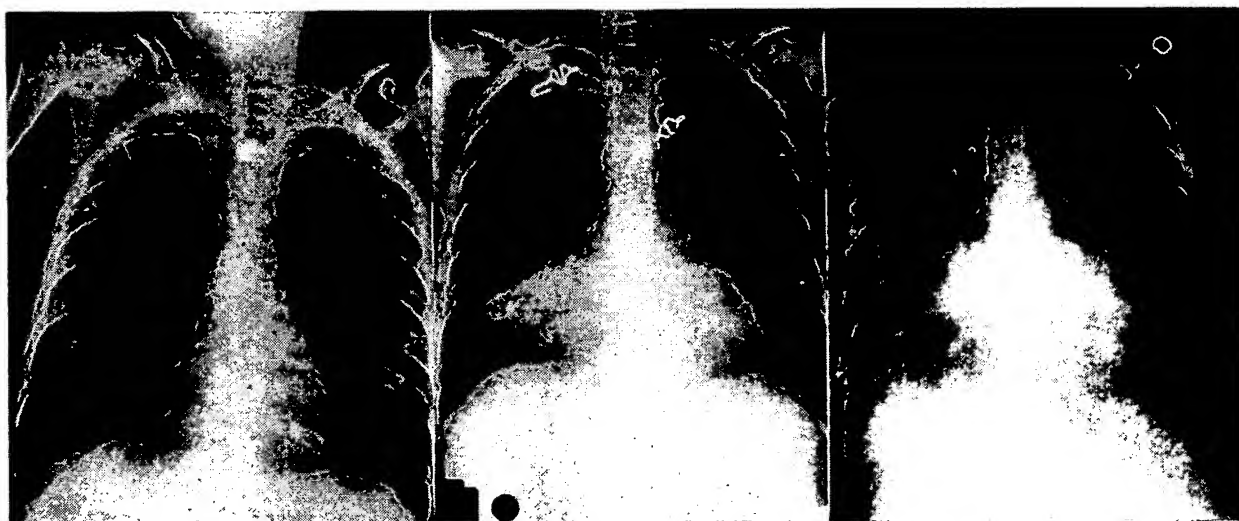


FIGURE 1. Chest roentgenograms taken on admission (1A, left), at the time pericarditis became evident (1B, center), and shortly before death (1C, right). Note progressive increase in RML infiltrate and cardiac silhouette caused by invasive aspergillosis.

geal ulcerations. The pain worsened over the next two days, and the patient developed a rapid and irregular pulse. A pericardial friction rub then was noted, and chest roentgenogram revealed that the RML infiltrate had increased and now was associated with an enlarged cardiac silhouette (Fig. 1, right). An ECG showed low voltage and atrial fibrillation, and echocardiogram demonstrated a pericardial effusion of approximately 50 ml.

The patient was taken to surgery, where the right side of the chest was entered through a parasternal incision. The RML was found to be firm and pale, the pericardium was covered with a shaggy exudate, and the pericardial sac contained 50 ml of serosanguinous fluid. A pericardial window was created, and the pericardium and RML were biopsied.

On microscopic examination, the RML tissue was hemorrhagic and infarcted. Branching, septate hyphae were seen on the pleural surface; sheets of the same hyphae were found adjacent to and invading the pericardium. Cultures of the pericardium and pericardial fluid subsequently grew *Aspergillus niger*.

Amphotericin B was continued to a total dose of 750 mg, and the patient's neutrophil count rose to 700/cu mm, indicating increased graft function. The patient was stable for two days until her cardiac silhouette enlarged even more on chest roentgenogram, and she developed a 30-mm paradoxical pulse (Fig 1, center). That night, the patient required endotracheal intubation and mechanical ventilation for worsening cardiopulmonary function. Pericardiectomy was planned, but she developed massive hemoptysis and died in ventricular fibrillation the following day.

At autopsy, the patient's pericardium, lungs, pleurae, heart, kidneys intestines and thyroid gland were found to be involved with *Aspergillus*. The fungus had invaded the aorta and the left main and left anterior descending coronary arteries, as well as the proximal right pulmonary arterial tree.

DISCUSSION

Invasive aspergillosis is second only to candidiasis of the fatal mycoses seen in cancer patients.³ Although it occurs most commonly in persons with neoplasms, aspergillosis also has been reported in immunosuppressed persons with sarcoidosis and collagen vascular diseases

and in individuals undergoing renal, cardiac, and bone marrow transplantation.¹⁻³ Factors predisposing to aspergillosis include granulocytopenia, broad spectrum antibiotic therapy, and the administration of corticosteroids.^{1,2} All of these factors were present in this patient.

Aspergillus characteristically enters the body through the respiratory tract. In the lungs, tissue invasion usually is manifested by bronchopneumonia or by a distinctive pattern of hemorrhagic infarction. Such infarction, which was seen in this patient, occurs secondary to vascular invasion by mycelial elements with thrombosis and occlusion of the pulmonary vessels. The fungus then may spread from the lungs by direct invasion, or more commonly, by hematologic dissemination.²

The organs most often involved in disseminated aspergillosis are the lungs, intestine, brain, kidneys, liver, esophagus, and heart, in that order. Pericardial involvement is unusual, occurring in only three of 93 patients in a series from the Memorial Sloan-Kettering Cancer Center and four of 98 patients from the National Cancer Institute (NCI).^{1,2} None of the NCI patients had signs or symptoms suggestive of pericarditis or pericardial tamponade.² In 1962, Fraumeni and Fear⁴ described a lymphoma patient with distended neck veins and pulsus paradoxus, but that is the only reported case in which *Aspergillus* caused an obvious pericarditis.

Even when the diagnosis of aspergillosis is made, treatment often is unsuccessful. Therapy includes the administration of amphotericin B, flucytosine, and aerosolized nystatin, either alone or in combination.^{3,6,7} Rifampin and amphotericin B also have been used together.⁸

According to one recent report, the clinical outcome of cancer patients with aspergillosis correlates best not with the total dose of amphotericin but with the recovery of circulating neutrophils.^{1,3} Outcome also has been reported to improve with early diagnosis and treatment.⁹ However, this patient already was receiving

amphotericin B and rifampin when her aspergillosis became apparent, and she had achieved partial bone marrow engraftment. Her case underscores the difficulty of diagnosing and treating invasive aspergillosis in immunocompromised hosts as well as the unusual manifestations of this disease.

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Spontaneous Return of Patency in a Completely Occluded Coronary Artery*

Neale D. Smith, M.D., and Henry DeMots, M.D.

We observed a 56-year-old man in whom an occluded right coronary artery was observed to be widely patent on a subsequent angiogram 40 months later. This "regression," which occurred without a change in his risk factors, shows that manipulation of risk factors can be proven to be a cause of regression only in controlled studies.

Serial coronary arteriography has shown that the expected course of coronary atherosclerosis is a steady and relentless progression of disease.¹⁻⁴ The rate of progression of an individual lesion is highly unpredictable but there appears to be a relationship between the number and severity of risk factors such as cigarette smoking, hypertension, and hyperlipoproteinemia and the rate of progression of coronary artery disease.^{1,5}

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Regression of atherosclerosis in animals^{6,7} and in the peripheral vessels in man has been shown in response to manipulation of the atherogenic stimuli.^{8,9} Because it is generally presumed that coronary artery disease always progresses, or that if regression is possible it occurs only after vigorous therapy of atherogenic stimuli, we are reporting a patient with a prior inferior myocardial infarction in whom angiographic "improvement" of a right coronary artery occlusion was demonstrated. The improvement occurred without treatment of known risk factors for coronary artery disease.

CASE REPORT

The patient is a 56-year-old man who presented to another hospital with acute chest pain, diaphoresis, and syncope in December 1974. The ECG showed evidence of an acute inferior myocardial infarction and complete heart block. He was treated with intravenous isoproterenol, atropine, and oral prednisone. Eight days after myocardial infarction, he had a right femoral artery embolus which was uneventfully removed.

After discharge from the hospital, he noted persistent chest pain and dyspnea on exertion. He was admitted for re-evaluation in April 1975.

He was 177.8 cm (70 inches) tall and weighed 72.3 kg (160 pounds). The arterial blood pressure was 115/80 mm Hg. The fasting blood sugar level was 90 mg/100 ml. A plasma cholesterol determination was not made.

Coronary arteriography was performed using the Judkins femoral percutaneous technique. Cineangiograms were made of the right and left coronary arteries in the right anterior oblique and left anterior oblique projections. Large film serial roentgenograms using a rapid film changer programmed for ten exposures in three seconds were made of each coronary artery in the lateral, right anterior oblique, and left anterior oblique projections. Secobarbital, 100 mg intramuscularly, was given prior to the procedure. Nitroglycerin 0.4 mg sublingual, was given prior to introduction of the right coronary artery catheter. The procedure was accomplished without complication; there was no angina during the procedure.

The examination revealed a complete occlusion in the midportion of the right coronary artery, complete occlusion of the left anterior descending coronary artery, and irregularity without significant obstruction in the proximal left circumflex coronary artery (Fig 1). There was complete morphologic consistency of all films. The left ventriculogram demonstrated inferior and apical akinesis and an ejection fraction of 0.15. Coronary bypass surgery was not recommended because of the poor ventricular function and because distal coronary arteries suitable for bypass grafting were not identified.

He was treated with progressively larger doses of propranolol until the dose of that medication reached 320 mg per day. Digoxin, 0.25 mg, was given daily and a thiazide diuretic was given for a brief period. No other drug therapy was used.

When the patient was first evaluated at our hospital in April 1978, he continued to complain of severe angina and dyspnea on exertion. In the interim, he had continued to smoke 1½ packs of cigarettes per day as he had done for the previous 30 years. He had not changed his diet. His disability was such that he engaged in minimal physical activity.

He weighed 168 pounds. His arterial blood pressure was 130/85 mm Hg. He had bibasilar rales, an S3, and pedal

Invasive aspergillosis presenting as pericarditis and cardiac tamponade

JM Luce, RC Ostenson, SC Springmeyer and LD Hudson

Chest 1979;76;703-705

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A M E R I C A N C O L L E G E O F



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Persistence, Replacement, and Microevolution of *Cryptococcus neoformans* Strains in Recurrent Meningitis in AIDS Patients

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Six separate human immunodeficiency virus-positive patients with cryptococcal meningitis were each found to have been infected with a unique strain of *Cryptococcus neoformans* on the basis of genomic DNA fingerprinting analysis with the microsatellite sequence-containing oligonucleotide probe (GGAT)₄ and by random amplification of polymorphic DNA. Two patients (A and B) experienced a recurrent episode of infection. Between 12 and 16 single-colony isolates recovered from primary isolation media (>50% of *C. neoformans* colonies recovered) from cerebrospinal fluid specimens were fingerprinted from both patients during each episode. The fingerprints of both isolate collections from patient B were very similar, although minor polymorphisms were evident in both sets of profiles. The fingerprints of the isolate collection from the initial episode of infection in patient A were also identical to each other, apart from minor polymorphisms, but they were clearly different from the corresponding profiles of the isolate collection from the recurrent episode, the latter of which were completely identical, apart from minor polymorphisms in a single isolate. Furthermore, prolonged storage and in vitro subculture of the isolates did not alter the fingerprint profiles. These results provided convincing evidence that patients A and B were each infected with a single *C. neoformans* strain during each episode of infection and that in patient B, the same strain persisted and caused both episodes, while in patient A, a different strain was responsible for each episode. The prevalence of polymorphisms in multiple single-colony isolates from both patients also suggested that *C. neoformans* populations may undergo microevolution.

Cryptococcus neoformans is an encapsulated basidiomycetous yeast species which occurs naturally in the environment and is frequently associated with pigeon droppings and soil contaminated with avian guano (11). Under most circumstances, inhalation of this organism fails to cause symptomatic infection (14). However, in 5 to 10% of individuals with AIDS, severe life-threatening disease in the form of meningoencephalitis can occur (6). The vast majority of the isolates responsible for these infections are *C. neoformans* var. *neoformans* serotype A (10).

Because of the perceived importance of these organisms as human pathogens, the species has been subjected to intense study during the last decade. In this regard, the recent development and application of techniques designed to differentiate between individual isolates are of particular relevance because of the high incidence of recurrent cryptococcal infections in AIDS patients once antifungal therapy has ceased (20). In order to design effective antifungal drug treatment regimens, it is important to determine if recurrent disease is the result of reinfection with the original strain or infection with a novel strain. Unfortunately, individual strains of *C. neoformans* are morphologically and physiologically indistinguishable, and isolates of *C. neoformans* var. *neoformans* can only be divided into three serotypes (serotypes A, D, and A-D) (9). Consequently, techniques used in the epidemiological analysis of these organisms have concentrated on detecting genetic differences between individual isolates. To date, a wide variety of molecular typing systems have been applied to *C. neoformans* epidemiol-

ogy, including electrophoretic karyotyping (17, 19), PCR fingerprinting (13), random amplified polymorphic DNA (RAPD) analysis (2, 8), multilocus enzyme typing (1, 2), allelic variation of the *URA5* locus (3, 5), and DNA fingerprinting with (i) genomic DNA probes (5, 18, 22, 23, 26, 27), (ii) mitochondrial DNA probes (25), and (iii) oligonucleotide probes homologous to microsatellite sequences (8). The general consensus from many of these studies is that *C. neoformans* strains exhibit considerable genetic heterogeneity and that recurrent infections are apparently due to the persistence of the original infecting strain (17, 22, 23). However, a recent study by Haynes et al. (8) indicated, on the basis of oligonucleotide and RAPD fingerprint analysis of genomic DNA, that in two patients (from five examined in total), recurrent infections may have been due to reinfection with a novel strain. In addition, this study indicated that one patient was coinfecting with more than one strain during a single episode of infection. These data have aroused some controversy (4), and their clinical implications are of sufficient importance to warrant the analysis of additional cases of recurrent *C. neoformans* infection in order to confirm unequivocally that reinfection with novel strains may occur. The purpose of this study was to corroborate these earlier findings by analyzing multiple single-colony isolates recovered from patients during two recurrent episodes of meningitis and to determine the extent of genetic diversity within phenotypically homogeneous populations of *C. neoformans* recovered from the same clinical specimen.

MATERIALS AND METHODS

***C. neoformans* isolates.** *C. neoformans* isolates were recovered from six human immunodeficiency virus-infected individuals with cryptococcal meningitis at The Chelsea and Westminster Hospital, London, United Kingdom, between January 1994 and April 1995. In the case of four of these patients, *C. neoformans* isolates were recovered from a single episode of infection. The remaining two patients,

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both of whom were homosexual males with AIDS, experienced two successive episodes of meningitis. These two patients, termed A and B, respectively, presented with the classical symptoms of cryptococcal meningitis for the first time in November 1994, and in both cases, *C. neoformans* was isolated by plating of aliquots of cerebrospinal fluid (CSF) on Sabouraud's agar. After treatment with 0.6 to 0.7 mg of amphotericin B $\text{kg}^{-1} \text{day}^{-1}$ for 2 to 4 weeks, both patients made a complete clinical recovery. Thereafter, each patient received 200 to 400 mg of fluconazole day^{-1} on an ongoing basis. However, clinical symptoms of meningitis reappeared in patient A in April 1995 and in patient B in March 1995, and in both cases, *C. neoformans* was again isolated from CSF samples. The time intervals between the initial and recurrent isolation of *C. neoformans* from patients A and B were 158 and 112 days, respectively. No additional specimens were taken from either patient during the symptom-free period between episodes of meningitis. Isolates were recovered on Sabouraud's agar after incubation at 37°C for 48 h and subcultured on fresh media prior to identification with the API ID 32C Yeast Identification System (bioMérieux, Marcy l'Etoile, France) and urea assimilation. Each isolate was stored in Protect cryo-storage vials (STC, Heywood, Lancashire, United Kingdom) at -20°C prior to detailed analysis. The CSF specimens from the initial and recurrent episodes of meningitis for both patients A and B each yielded between 10 and 30 *C. neoformans* CFU on primary isolation media. In the case of both episodes of infection in patient A and in the recurrent episode of infection in patient B, 20 individual well-separated *C. neoformans* colonies from the primary isolation plates were selected at random and stored; 16 of these from each isolate collection were subjected to further detailed analysis. In the case of the initial episode of infection in patient B, only 12 *C. neoformans* CFU were recovered on primary isolation media, all of which were stored and subjected to detailed analysis. The isolate collections from the initial episodes of infection in patients A and B were labelled A_i1-20 and B_i1-12, respectively, while those from the recurrent episodes were labelled A_r1-20 and B_r1-20, respectively.

Fluconazole susceptibility testing. The susceptibility of *C. neoformans* clinical isolates to fluconazole was determined by broth microdilution in RPMI 1640 medium (15). Isolates were grown in 96-well microtiter plates (Corning) incubated at 35°C for 72 h with agitation (16). An end point of 80% growth inhibition (IC_{80}) was determined for each isolate by measuring the A_{405} with an automated microplate reader (Spectra I; SLT-Labinstruments, Salzburg, Austria).

DNA fingerprinting. *C. neoformans* total cellular DNA was purified as described previously (22). Restriction fragments generated by digesting total cellular DNA samples to completion with the restriction enzyme *Eco*RI (Promega Corp., Madison, Wis.) were separated by electrophoresis through 0.8% (wt/vol) agarose gels and transferred onto nylon membrane filters (MSI, Westboro, Mass.) according to the method of Southern (21). The oligonucleotide probe (GGAT)₄ was end labelled with [γ -³²P]dATP (Amersham International Plc., Little Chalfont, Buckinghamshire, United Kingdom [$>5,000 \text{ Ci mmol}^{-1}$]) and T4 polynucleotide kinase (Promega Corp.) according to the manufacturer's instructions. Labelled (GGAT)₄ was hybridized to the digested DNA as described previously (24).

RAPD. PCRs were performed in a final volume of 25 μl containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 3.0 mM MgCl₂; 200 μM (each) dATP, dCTP, dTTP, and dGTP (Promega Corp.); 20 pM primer; 0.5 U of *Taq* DNA polymerase (Promega Corp.); and approximately 10 ng of *C. neoformans* total genomic DNA. The following oligonucleotide primers were used: 1, 5'-GCGATCCCCA3'; 2, 5'-(GATA)₃3'; and 3, 5'-AACGCGCAAC3'. Amplification reactions were performed in a Perkin-Elmer Cetus DNA thermal cycler under the following conditions: 94°C for 5 min, 36°C for 5 min, and 72°C for 5 min for 4 cycles followed by 30 cycles at 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. This was followed by an incubation period of 72°C for 10 min. After amplification, 20- μl aliquots of the reaction mixtures were electrophoresed in 1.5% (wt/vol) agarose gels, and the amplified products were visualized under UV light after being stained with ethidium bromide.

RESULTS

Oligonucleotide fingerprinting of *C. neoformans* isolates. Total cellular DNA was purified from a single isolate of *C. neoformans* recovered in each case from CSF, skin, and blood samples obtained from two patients with meningitis and from CSF cultures recovered from another four patients suffering from meningitis. Aliquots of DNA from these isolates were digested to completion with *Eco*RI, and the resulting fragments were separated by agarose gel electrophoresis and transferred to nylon membrane filters prior to hybridization analysis with the ³²P-labelled oligonucleotide probe (GGAT)₄. The fingerprints generated from isolates recovered from different anatomical sites in the same patient were found to be indistinguishable. However, the fingerprints generated from isolates recovered from separate patients were each found to be very

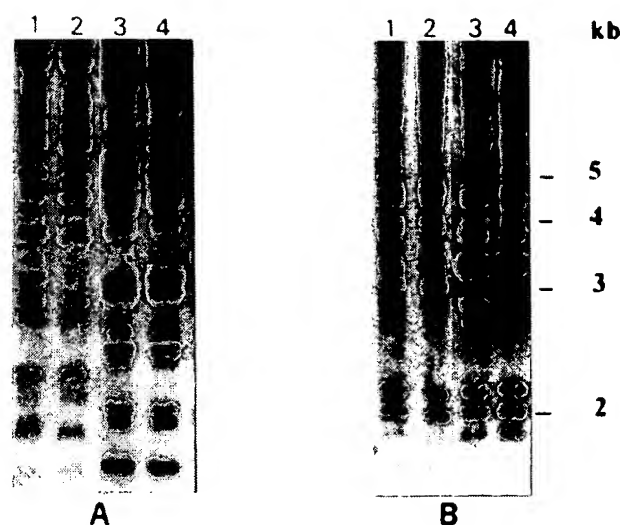


FIG. 1. Autoradiogram of *Eco*RI-digested total cellular DNA from single-colony isolates of *C. neoformans* recovered from CSF specimens from patients A and B during two successive episodes of meningitis and hybridized with the oligonucleotide probe (GGAT)₄. Lanes 1 and 2 and 3 and 4 show fingerprint profiles generated from two single-colony isolates recovered from the same CSF sample during the initial and recurrent episodes of infection, respectively. (A) Lanes 1 to 4 show profiles generated from isolates A_i1, A_i2, A_r11, and A_r12, respectively. (B) Lanes 1 to 4 show profiles generated from isolates B_i1, B_i2, B_r11, and B_r12, respectively. The relative positions of molecular size standards are indicated to the right.

different (data not shown), indicating that each patient was infected with a unique strain of *C. neoformans*.

Total cellular DNA was purified from three single-colony isolates from the initial and recurrent isolate collections recovered from two AIDS patients (A and B), each of whom experienced two successive episodes of meningitis, and DNA fingerprints were generated as described above. In the case of patient A, the hybridization profiles obtained with the three isolates recovered during the initial episode of meningitis (data for isolates A_i1 and A_i2 are shown in Fig. 1A, lanes 1 and 2) were identical to each other but were clearly significantly different from the corresponding profiles obtained with the three isolates (also identical to each other) recovered during the recurrent episode of infection (data for isolates A_r11 and A_r12 are shown in Fig. 1A, lanes 3 and 4). In contrast, the hybridization patterns obtained with both sets of isolates recovered from patient B were found to be very similar, but some band differences were evident. For example, there was a hybridization band with a size of approximately 3 kb, which was present in the profiles of the three isolates from the initial episode of infection (data for isolates B_i1 and B_i2 are shown in Fig. 1B, lanes 1 and 2) but was absent in the corresponding profiles of the three isolates from the recurrent episode (data for isolates B_r11 and B_r12 are shown in Fig. 1B, lanes 3 and 4). Direct visual analysis of ethidium bromide-stained agarose gels containing separated *Eco*RI-generated fragments of genomic DNA from the isolates concerned showed that the restriction fragment length polymorphism patterns of the recurrent isolates from patient B lacked a heavily stained band, also approximately 3 kb in size, that was present in the restriction fragment length polymorphism patterns of the three isolates from the initial episode of infection (data not shown). Furthermore, an additional hybridization band with a size of approximately 2.8 kb was present in the profile of isolate B_r11 which was not present in the profile of isolate B_r12 (Fig. 1B, lanes 3 and 4).

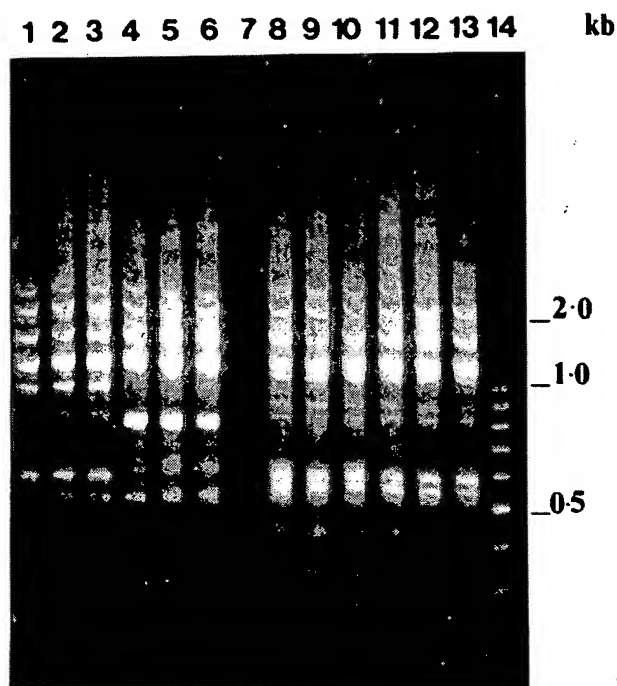


FIG. 2. RAPD products from single-colony isolates of *C. neoformans* recovered from CSF specimens from patients A and B during two successive episodes of meningitis and amplified with oligonucleotide primer 1 (5'GCGATCCCC A3'). Lanes 1 to 3 (isolates A_I1 to 3) and 8 to 10 (isolates B_I1 to 3) show RAPD profiles generated from three single-colony isolates recovered from the same CSF sample from patients A and B, respectively, during the initial episodes of infection. Lanes 4 to 6 (isolates A_{II}1 to 3) and 11 to 13 (isolates B_{II}1 to 3) show RAPD profiles generated from three single-colony isolates recovered from the same CSF sample from patients A and B, respectively, during recurrent episodes of infection. Lane 14, 100-bp DNA ladder size standards (Promega).

The fingerprint profiles of the isolates described above were found to be stable after repeated subculture (minimum of eight times) of the isolates over a 6-month period. During this time, total cellular DNA was prepared from each isolate on three separate occasions, and in each instance, fingerprint profiles identical to those shown in Fig. 1 were obtained with (GGAT)₄.

RAPD analysis of recurrent isolates. The RAPD profiles generated with primer 1 and target DNA from three single-colony isolates from the same clinical specimen examined from the initial episode of infection in patient A (A_I1, A_I2, and A_I3) were identical to each other (Fig. 2, lanes 1 to 3). Similarly, the corresponding profiles obtained with the target DNA from an additional three single-colony isolates from the same clinical specimen recovered during the recurrent episode of infection (A_{II}1, A_{II}2, and A_{II}3 [Fig. 2, lanes 4 to 6]) were also found to be identical to each other. However, the patterns obtained from both sets of isolates were totally distinct. In contrast, the RAPD patterns generated with the three isolates examined from both the initial and recurrent episodes of meningitis in patient B (B_I1, B_I2, B_I3, B_{II}1, B_{II}2, and B_{II}3) were all found to be identical (Fig. 2, lanes 8 to 13). Additional RAPD experiments with the same target DNA as that used in the experiments described above and primers 2 and 3 also generated distinct profiles for the three A_I and the three A_{II} isolates and were unable to discriminate between the three B_I and the three B_{II} isolates. RAPD fingerprint profiles were also found to be reproducible after repeated subculture of the isolates with target DNA prepared on three separate occasions.

Analysis of multiple single-colony isolates. In order to investigate the possibility that patients A and B may have been infected with more than one strain of *C. neoformans* during both episodes of meningitis, total cellular DNA was prepared from an additional 13 single-colony isolates (9 in the case of the initial episode of infection in patient B) recovered, in each case, from the same clinical specimens as the isolates used in the experiments described above, and then hybridization fingerprints were generated with (GGAT)₄. For both patients, only a single fingerprint pattern was found for each group of single-colony isolates examined. As in the results described above, the patterns of the 13 additional A_I isolates differed considerably from those of the 13 additional A_{II} isolates, while those of the 9 additional B_I isolates were almost identical to those of the 13 additional B_{II} isolates. However, whereas the A_{II} isolates all yielded identical fingerprint patterns (fingerprints from 14 isolates are shown in Fig. 3a), the corresponding profiles of the A_I, B_I, and B_{II} isolate collections, respectively, although essentially homogeneous, showed numerous subtle but distinct minor polymorphisms. An example of the profiles obtained with the B_{II} isolate collection is shown in Fig. 3b.

RAPD fingerprints of the additional single-colony isolates from each isolate collection from patients A and B were also generated with primer 2 and primer 3, respectively. The results confirmed the findings obtained in the experiments described above with three single-colony isolates from each isolate collection. However, minor polymorphisms were evident in some of the RAPD profiles of all four single-colony isolate collections, including one single-colony isolate from the recurrent episode of infection in patient A (Fig. 4).

Fluconazole susceptibility of *C. neoformans* isolates. The fluconazole susceptibilities of isolates 1 to 3 from the initial and recurrent isolate collections for both patients A and B were determined by broth microdilution. Isolates A_I1 to 3, B_I1 to 3, and B_{II}1 to 3 yielded an IC₈₀ of 32 µg ml⁻¹, whereas isolates A_{II}1 to 3 yielded an IC₈₀ of 8 µg ml⁻¹.

DISCUSSION

Only a small number of studies have focused on the relationship between isolates of *C. neoformans* from successive episodes of meningitis in individual patients (8, 17, 22, 23). Some of these studies have indicated that relapse of cryptococcal meningitis is due to the persistence of the originally infecting strain (17, 22, 23), whereas another study has shown that in two separate individuals, recurrence of infection was apparently due to reinfection with a novel strain (8). In addition, the latter study provided evidence that one patient was infected with more than one *C. neoformans* strain during a single episode of meningitis. The present study was undertaken to confirm these findings by analysis of multiple *C. neoformans* single-colony isolates recovered from the same clinical specimen obtained from two patients during each of two successive episodes of disease. Fingerprint analysis of genomic DNA with the microsatellite sequence-containing oligonucleotide probe (GGAT)₄ and RAPD analysis with three separate oligonucleotide primers showed that the six patients included in the study were each infected by different *C. neoformans* strains. This is in agreement with earlier studies which described the genetic heterogeneity of serotype A *C. neoformans* strains (3, 7, 8, 17, 26, 27). In addition, in the case of the two patients from which *C. neoformans* isolates were recovered from specimens taken from different anatomical sites during the same episode of meningitis, all of the isolates from the same individual yielded the same fingerprint pattern.

Two of the six patients under study (A and B) suffered

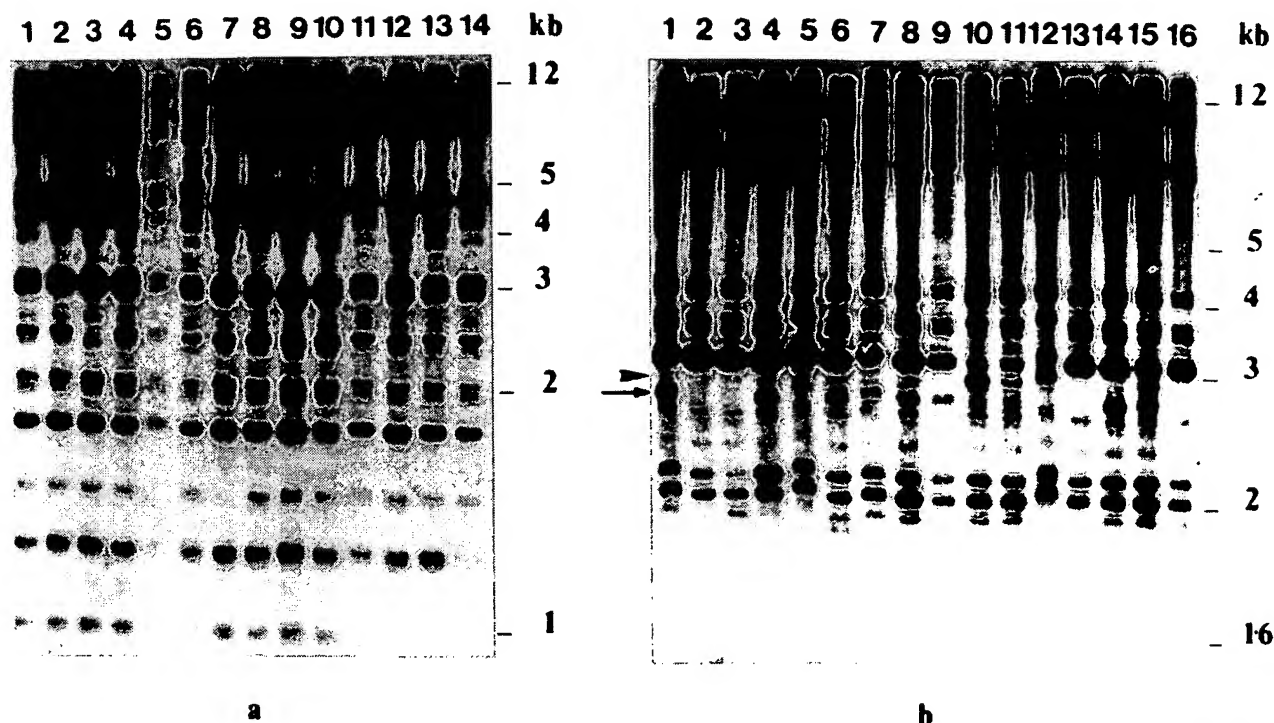


FIG. 3. Autoradiograms of *Eco*RI-digested total cellular DNA from *C. neoformans* single-colony isolates recovered from the same CSF specimen in the case of patients A and B, respectively, during the recurrent episode of meningitis after hybridization analysis with the oligonucleotide probe (GGAT)₄. (a) Profiles shown in lanes 1 to 14 were from single-colony isolates A₁₁1 to A₁₁14, respectively. (b) Profiles shown in lanes 1 to 16 were from single-colony isolates B₁₁1 to B₁₁16, respectively. The arrow and arrowhead shown to the left of panel b indicate the relative positions of polymorphic hybridization bands with sizes of approximately 2.6 and 2.9 kb present in isolate profiles shown in lanes 1, 4, 6, 7, 8, 9, 14, and 15 and lanes 10, 11, 12, and 15, respectively.

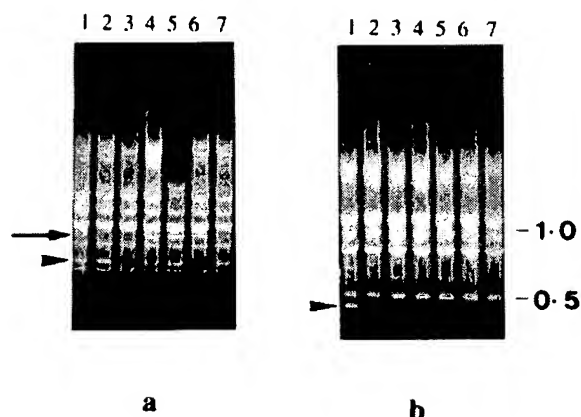


FIG. 4. Amplified RAPD products from single-colony isolates of *C. neoformans* recovered from CSF specimens from patient A during the initial and recurrent episodes of infection. (a) Profiles shown in lanes 1 to 7 were from single-colony isolates (A₁5 to A₁11) from the same specimen recovered during the initial episode of infection. (b) Profiles shown in lanes 1 to 7 were from single-colony isolates (A₁₁1 to A₁₁7) from the same specimen recovered during the recurrent episode of infection. The positions of the molecular size reference markers indicated to the right are in kilobase pairs. The arrowhead and arrow shown to the left of panel a indicate the relative positions of polymorphic bands with sizes of approximately 0.7 and 0.9 kb present in isolate profiles shown in lanes 1, 2, 4, 5, and 7 and 4, 5, 6, and 7, respectively. The arrowhead to the left of panel b indicates the position of a single polymorphic band with a size of approximately 0.45 kb present only in lane 1.

relapses in infection within 6 months of clinical resolution of the symptoms after therapy. Preliminary experiments were performed with three single-colony isolates recovered from CSF specimens from patients A and B during each episode of disease by both DNA fingerprinting techniques (Fig. 1 and 2). The results showed that the (GGAT)₄-generated hybridization patterns obtained with the three single-colony isolates from the initial episode of meningitis in patient A, although identical to each other, were significantly different from the corresponding profiles of the three single-colony isolates recovered during the recurrent episode of disease in the same patient. In fact, the two sets of profiles, each of which contained ≥ 15 clearly resolved hybridization bands, shared no bands in common, strongly suggesting that patient A was infected with unrelated *C. neoformans* strains during each episode of meningitis. In contrast, the hybridization patterns obtained with the three single-colony isolates from both the initial and recurrent episodes of infection in patient B were very similar, with the majority ($\sim 75\%$) of bands shared in common. These results suggested that the same strain of *C. neoformans* was responsible for both episodes of disease. This also suggested that the recurrent episode of infection was due to persistence of the strain responsible for the initial episode in patient B. However, the possibility that patient B was reinfected with the same strain from an environmental source cannot be discounted.

Fluconazole susceptibility data obtained from three single-colony isolates from each episode of meningitis in both patients A and B showed that isolates from the recurrent episode in patient A had a fourfold lower ($8 \mu\text{g ml}^{-1}$) fluconazole IC₈₀ than that obtained with isolates from the initial episode ($32 \mu\text{g ml}^{-1}$). In contrast, all six isolates tested from patient B yielded

the same fluconazole IC_{80} ($32 \mu\text{g ml}^{-1}$). These results strengthen the conclusions derived by fingerprinting analysis that the recurrent episode of meningitis in patient A was caused by a novel strain, while both episodes in patient B were caused by the same strain.

On the basis of the results described above, it was still possible that both patients A and B were infected with two or more strains of *C. neoformans* during each episode of disease and that this was not reflected in the single-colony isolates tested from primary isolation plates. If two strains were present during a particular episode of infection in different relative abundance, the chance of detecting a particular strain would directly reflect the number of single-colony isolates tested from the primary isolation plates. A strain present in low abundance relative to a second strain during a particular episode of disease in a given patient might not be detected if only a few colonies were sampled. A change in the relative abundance of the two strains during a subsequent episode of disease, assuming that both strains persisted between episodes, could lead to the conclusion that the recurrent episode was due to a novel strain. To unequivocally determine the presence of multiple strains during a specific episode of infection would require analysis of all of the *C. neoformans* colonies recovered on primary isolation. However, from a routine perspective, this would clearly not be practical for logistical reasons. To improve the chances of detecting the presence of more than one *C. neoformans* strain from each of the specimens obtained during both episodes of infection in patient A and from the recurrent episode of infection in patient B, a total of 16 single-colony isolates from the primary isolation plates were analyzed (i.e., an additional 13 single-colony isolates from each specimen). This represented >50% of the total number of *C. neoformans* colonies recovered from the original specimens in each case. In the case of the initial episode of infection in patient B, all 12 (i.e., 100%) of the *C. neoformans* CFU recovered on primary isolation were analyzed. Fingerprinting analysis of the additional single-colony isolates from the initial and recurrent specimens from patients A and B confirmed the preliminary findings described above with each of three single-colony isolates from each episode of infection. The very close similarity of the fingerprint patterns obtained with the initial and recurrent isolate collections from patient B makes it unlikely that this patient was infected by more than one strain of *C. neoformans* during each episode of meningitis and suggests that both disease episodes were caused by the same strain. Furthermore, although the fingerprint patterns obtained with the single-colony isolate collections from the initial and recurrent episodes of disease in patient A were totally different from each other, the profiles obtained with the 16 isolates comprising each collection were remarkably homogeneous, apart from minor polymorphisms evident in patterns from isolates from the initial episode. These findings and the confirmatory data obtained by RAPD analysis provided strong evidence that patient A was infected with a single strain of *C. neoformans* during each episode of disease and that a different strain was responsible for each episode.

As far as we are aware, the present work and another recent report from our laboratories (8) are the only studies which have indicated that recurrent episodes of meningitis in the same patient can be caused by different strains of *C. neoformans*. The only other published studies on this subject indicate that recurrent episodes of meningitis in the same patient are due to persistence of the same *C. neoformans* strain responsible for the initial episode of disease (17, 22, 23). There are several possible reasons for the disparity between our results and those of previous studies, including different patient man-

agement and treatment regimens. However, the use of different fingerprinting techniques in the various studies to distinguish between isolates and their relative discriminatory powers is probably more important. Each of the fingerprinting probes employed to date recognizes different genetic markers and is likely to have a different discriminatory ability.

Failure to detect minor polymorphisms in the single-colony isolate collection from the recurrent episode of infection in patient A by fingerprinting with the (GGAT)₄ probe was surprising, given that they were readily detectable in both isolate collections from patient B and in the initial isolate collection from patient A (Fig. 4a, lane 1). However, minor polymorphisms were evident in the RAPD profiles of one of the 16 single-colony isolates from the recurrent episode of meningitis in patient A. It is possible that some strains of *C. neoformans* are inherently more unstable or genetically pleomorphic and thus are more prone to generate polymorphisms than others. In order to investigate whether the polymorphisms were due to genetic instability in vitro, three of the single-colony isolates from each isolate collection recovered from patients A and B were subcultured at least eight times over a 6-month period, and fingerprinting experiments were repeated on three separate occasions with freshly prepared total cellular DNA. The results demonstrated that the fingerprint profiles of the single-colony isolates were reproducible. Furthermore, storage of isolates for a period of at least 6 months at -20°C did not detectably affect the fingerprint profiles. All of these results demonstrated that the generation of polymorphisms was not due to genetic instability in vitro; the generation of polymorphisms is more likely to be a response to unfavorable environmental conditions in vivo. Similar polymorphisms in *Candida albicans* DNA fingerprint profiles have recently been described and have been attributed to the occurrence of microevolution (12).

It has been suggested that *C. neoformans* strains only rarely reproduce sexually and that clinical populations of the organism are usually clonal in origin (5, 14). In the absence of genetic exchange through sexual reproduction, microevolution could conceivably confer a selective advantage under unfavorable environmental conditions.

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Invasive aspergillosis presenting as pericarditis and cardiac tamponade

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A M E R I C A N C O L L E G E O F



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occurred in association with probable pneumoconiosis: Carasso et al³ reported a 48-year-old woman with a bronchoesophageal fistula due to silicotic mediastinal lymphadenopathy, and Frew⁴ reported a 51-year-old man with a chest roentgenogram suggestive of pneumoconiosis and dysphagia due to a tuberculoma in the esophagus. Mediastinal lymph node enlargement due to other granulomatous diseases such as sarcoidosis and histoplasmosis have been shown to compress the esophagus.¹⁻²

Although this patient had no specific signs or symptoms of tuberculosis, tissue cultures of the cervical lymph nodes grew *M intracellulare*. The increased incidence of *Mycobacterium tuberculosis* in silicotic patients is well known. Also atypical mycobacterial infections appear to be more frequent in patients with pneumoconioses.⁵⁻⁶ Blacks generally have a lower incidence of clinical atypical mycobacteriosis than whites, but our patient's silicosis and diabetes mellitus may have increased his risk to infection.⁵ His anemia of chronic disorders may be related to the mycobacteriosis.⁷

Although this patient was lost to follow up by the chest service, it is interesting to speculate what the most appropriate course of therapy for him would have been. We recommended that he undergo a thoracotomy to remove the mediastinal lymph nodes compressing the esophagus and perhaps reduce the burden of tuberculous tissue as well. We also would have administered chemotherapy with five to six antituberculosis drugs because *M intracellulare* is notoriously resistant to conventional therapy.⁸ Atypical mycobacterioses are even more difficult to treat in patients with pneumoconioses.⁶

This is the first reported case, to our knowledge, in which silicotic lymphadenopathy complicated by *M intracellulare* infection produced dysphagia by extrinsic compression of the esophagus. Silicosis should be considered among other granulomatous diseases such as mediastinal granuloma, tuberculosis, and sarcoidosis in the differential diagnosis of dysphagia.

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Invasive Aspergillosis Presenting as Pericarditis and Cardiac Tamponade*

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A 38-year-old leukemic patient developed pericarditis and cardiac tamponade due to *Aspergillus niger* one month after undergoing bone marrow transplantation. She failed to improve even though amphotericin B and rifampin therapy had been initiated before infection was evident. Her unique case illustrates both the unusual presentations of invasive aspergillosis and the difficulty of diagnosing and treating this increasingly common disease

Invasive aspergillosis is recognized with increased frequency among immunocompromised patients.¹⁻³ However, recognition often is delayed by the unusual and nonspecific manifestations of this condition. We present the case of a bone marrow transplant recipient in whom pericarditis and cardiac tamponade were the first clinical indications of invasive aspergillosis.

CASE REPORT

The patient was a 38-year-old woman with acute myelomonocytic leukemia who was transferred to the Fred Hutchinson Cancer Research Center (FHCRC; UPN807) in second relapse. Previous chemotherapy had included cytarabine, 6-thioguanine, and daunomycin. Admission laboratory findings included pancytopenia with a peripheral neutrophil count of 150/cu mm. Chest roentgenogram and ECG were normal (Fig 1, left). The patient was placed in laminar air flow and was given prophylactic oral antibiotics. She was prepared for transplantation with 1 mg/kg/body weight of nitrogen mustard followed by 1,200 rads of total body irradiation in six divided doses, as per FHCRC protocol.⁴

During preparation, the patient was given intravenous carbenicillin and gentamicin for fever. Intravenous amphotericin B, 25 mg/day, and rifampin, 300 mg/day, were added when blood cultures grew *Candida tropicalis*. Two weeks after admission, the patient received a bone marrow transplant from her HLA-matched sibling, who also was the source of daily granulocyte infusions.

The patient's posttransplantation course was complicated by an episode of pulmonary edema which was thought to be due to pre-existent anthracycline cardiac toxicity and fluid overload. Her respiratory status improved with diuretics, and her chest roentgenogram cleared but for a small right middle lobe (RML) infiltrate. She then developed toxic enteritis which was treated with intravenously administered corticosteroids.

Three weeks after admission, the patient noted anterior chest pain which radiated into her throat and was intensified in the supine position. The pain was felt to be due to *Candida* esophagitis after barium swallow disclosed esopha-

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FIGURE 1. Chest roentgenograms taken on admission (1A, left), at the time pericarditis became evident (1B, center), and shortly before death (1C, right). Note progressive increase in RML infiltrate and cardiac silhouette caused by invasive aspergillosis.

geal ulcerations. The pain worsened over the next two days, and the patient developed a rapid and irregular pulse. A pericardial friction rub then was noted, and chest roentgenogram revealed that the RML infiltrate had increased and now was associated with an enlarged cardiac silhouette (Fig. 1, right). An ECG showed low voltage and atrial fibrillation, and echocardiogram demonstrated a pericardial effusion of approximately 50 ml.

The patient was taken to surgery, where the right side of the chest was entered through a parasternal incision. The RML was found to be firm and pale, the pericardium was covered with a shaggy exudate, and the pericardial sac contained 50 ml of serosanguinous fluid. A pericardial window was created, and the pericardium and RML were biopsied.

On microscopic examination, the RML tissue was hemorrhagic and infarcted. Branching, septate hyphae were seen on the pleural surface; sheets of the same hyphae were found adjacent to and invading the pericardium. Cultures of the pericardium and pericardial fluid subsequently grew *Aspergillus niger*.

Amphotericin B was continued to a total dose of 750 mg, and the patient's neutrophil count rose to 700/cu mm, indicating increased graft function. The patient was stable for two days until her cardiac silhouette enlarged even more on chest roentgenogram, and she developed a 30-mm paradoxical pulse (Fig 1, center). That night, the patient required endotracheal intubation and mechanical ventilation for worsening cardiopulmonary function. Pericardiectomy was planned, but she developed massive hemoptysis and died in ventricular fibrillation the following day.

At autopsy, the patient's pericardium, lungs, pleurae, heart, kidneys intestines and thyroid gland were found to be involved with *Aspergillus*. The fungus had invaded the aorta and the left main and left anterior descending coronary arteries, as well as the proximal right pulmonary arterial tree.

DISCUSSION

Invasive aspergillosis is second only to candidiasis of the fatal mycoses seen in cancer patients.³ Although it occurs most commonly in persons with neoplasms, aspergillosis also has been reported in immunosuppressed persons with sarcoidosis and collagen vascular diseases

and in individuals undergoing renal, cardiac, and bone marrow transplantation.¹⁻³ Factors predisposing to aspergillosis include granulocytopenia, broad spectrum antibiotic therapy, and the administration of corticosteroids.^{1,2} All of these factors were present in this patient.

Aspergillus characteristically enters the body through the respiratory tract. In the lungs, tissue invasion usually is manifested by bronchopneumonia or by a distinctive pattern of hemorrhagic infarction. Such infarction, which was seen in this patient, occurs secondary to vascular invasion by mycelial elements with thrombosis and occlusion of the pulmonary vessels. The fungus then may spread from the lungs by direct invasion, or more commonly, by hematologic dissemination.³

The organs most often involved in disseminated aspergillosis are the lungs, intestine, brain, kidneys, liver, esophagus, and heart, in that order. Pericardial involvement is unusual, occurring in only three of 93 patients in a series from the Memorial Sloan-Kettering Cancer Center and four of 98 patients from the National Cancer Institute (NCI).^{1,2} None of the NCI patients had signs or symptoms suggestive of pericarditis or pericardial tamponade.² In 1962, Fraumeni and Fear⁵ described a lymphoma patient with distended neck veins and pulsus paradoxus, but that is the only reported case in which *Aspergillus* caused an obvious pericarditis.

Even when the diagnosis of aspergillosis is made, treatment often is unsuccessful. Therapy includes the administration of amphotericin B, flucytosine, and aerosolized nystatin, either alone or in combination.^{3,6,7} Rifampin and amphotericin B also have been used together.⁸

According to one recent report, the clinical outcome of cancer patients with aspergillosis correlates best not with the total dose of amphotericin but with the recovery of circulating neutrophils.^{1,3} Outcome also has been reported to improve with early diagnosis and treatment.⁹ However, this patient already was receiving

amphotericin B and rifampin when her aspergillosis became apparent, and she had achieved partial bone marrow engraftment. Her case underscores the difficulty of diagnosing and treating invasive aspergillosis in immunocompromised hosts as well as the unusual manifestations of this disease.

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Spontaneous Return of Patency in a Completely Occluded Coronary Artery*

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We observed a 56-year-old man in whom an occluded right coronary artery was observed to be widely patent on a subsequent angiogram 40 months later. This "regression," which occurred without a change in his risk factors, shows that manipulation of risk factors can be proven to be a cause of regression only in controlled studies.

Serial coronary arteriography has shown that the expected course of coronary atherosclerosis is a steady and relentless progression of disease.¹⁻⁴ The rate of progression of an individual lesion is highly unpredictable but there appears to be a relationship between the number and severity of risk factors such as cigarette smoking, hypertension, and hyperlipoproteinemia and the rate of progression of coronary artery disease.^{1,5}

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Regression of atherosclerosis in animals^{6,7} and in the peripheral vessels in man has been shown in response to manipulation of the atherogenic stimuli.^{8,9} Because it is generally presumed that coronary artery disease always progresses, or that if regression is possible it occurs only after vigorous therapy of atherogenic stimuli, we are reporting a patient with a prior inferior myocardial infarction in whom angiographic "improvement" of a right coronary artery occlusion was demonstrated. The improvement occurred without treatment of known risk factors for coronary artery disease.

CASE REPORT

The patient is a 56-year-old man who presented to another hospital with acute chest pain, diaphoresis, and syncope in December 1974. The ECG showed evidence of an acute inferior myocardial infarction and complete heart block. He was treated with intravenous isoproterenol, atropine, and oral prednisone. Eight days after myocardial infarction, he had a right femoral artery embolus which was uneventfully removed.

After discharge from the hospital, he noted persistent chest pain and dyspnea on exertion. He was admitted for reevaluation in April 1975.

He was 177.8 cm (70 inches) tall and weighed 72.3 kg (160 pounds). The arterial blood pressure was 115/80 mm Hg. The fasting blood sugar level was 90 mg/100 ml. A plasma cholesterol determination was not made.

Coronary arteriography was performed using the Judkins femoral percutaneous technique. Cineangiograms were made of the right and left coronary arteries in the right anterior oblique and left anterior oblique projections. Large film serial roentgenograms using a rapid film changer programmed for ten exposures in three seconds were made of each coronary artery in the lateral, right anterior oblique, and left anterior oblique projections. Secobarbital, 100 mg intramuscularly, was given prior to the procedure. Nitroglycerin 0.4 mg sublingual, was given prior to introduction of the right coronary artery catheter. The procedure was accomplished without complication; there was no angina during the procedure.

The examination revealed a complete occlusion in the midportion of the right coronary artery, complete occlusion of the left anterior descending coronary artery, and irregularity without significant obstruction in the proximal left circumflex coronary artery (Fig 1). There was complete morphologic consistency of all films. The left ventriculogram demonstrated inferior and apical akinesis and an ejection fraction of 0.15. Coronary bypass surgery was not recommended because of the poor ventricular function and because distal coronary arteries suitable for bypass grafting were not identified.

He was treated with progressively larger doses of propranolol until the dose of that medication reached 320 mg per day. Digoxin, 0.25 mg, was given daily and a thiazide diuretic was given for a brief period. No other drug therapy was used.

When the patient was first evaluated at our hospital in April 1978, he continued to complain of severe angina and dyspnea on exertion. In the interim, he had continued to smoke 1½ packs of cigarettes per day as he had done for the previous 30 years. He had not changed his diet. His disability was such that he engaged in minimal physical activity.

He weighed 168 pounds. His arterial blood pressure was 130/85 mm Hg. He had bibasilar rales, an S3, and pedal

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Colonization and Infection of Newborn Infants Caused by Bacteriophage-Group II *Staphylococcus aureus* Strains

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A staphylococcal strain which exhibited weak lytic reaction with group II phages was isolated from a newborn infant with a skin infection. Subsequent investigations established that this weakly reacting strain was responsible for an endemic level of infection and colonization within the hospital nursery. The use of consistently appearing weak lytic reactions in the evaluation of this episode is described.

Staphylococcus disease in hospital nurseries is a continuing concern to those responsible for infection control. The approach used in monitoring such infections includes mandatory culture of all infections, e.g., wounds, rash, pustules, etc., and routine culture of the cord and external nares. *Staphylococcus aureus* strains recovered

usually in a nursery and recovered sporadically from a series of infections and colonizations of newborn infants. The nursery had 31 operating bassinets with an average of 71 admissions per month during the period of study. This report briefly describes the clinical-epidemiological and laboratory features of this episode.

TABLE 1. Occurrence and phage type of group II *S. aureus* strains among newborn infants, U.S. Air Force Medical Center, Keesler Air Force Base, Miss. (January to August 1978)

Patient	Specimen source	Culture date	Phage type	
			RTD	100 × RTD
A	Pustular rash	9 February	NT ^a	3A ⁺ /3C ⁺
B	Blood	23 March	NT	3C ⁺
	Skin lesions	27 March	NT	3C ⁺
C	Nasal (colonization)	3 April	NT	3C ⁺
	Bullous lesions	3 April	NT	3C ⁺
D	Skin lesions	19 April	NT	3C ⁺ /55 ⁺
E	Bullous lesions	28 April	NT	3C ⁺ /55 ⁺
F	Bullous lesions	19 April	NT	3C ⁺
	Chest tube drainage (colonization)	5 May	NT	3C ⁺⁺
G	Nasal (colonization)	23 June	NT	3C ⁺
H	Cord (colonization)	28 June	NT	3C ⁺
I	Nasal (colonization)	28 June	NT	3C ⁺
	Bullous lesions	28 June	NT	3C ⁺
J	Eye drainage	12 July	NT	3C ⁺
K	Nasal lesions	9 August	3A ⁺	3A ⁺ /3C ⁺⁺

^a NT, Nontypable

from such infections are usually tested for antimicrobial susceptibility and are often phage typed as part of a surveillance program. At a large Air Force medical center, we encountered an unusual situation in which a "weakly typing" group II *S. aureus* strain was maintained endem-

As a part of the hospital infection control program at the U.S. Air Force Medical Center, Keesler Air Force Base, Miss., *S. aureus* isolates recovered from hospitalized patients with infections are periodically forwarded to the U.S. Air Force School of Aerospace Medicine's Epidemiology Division laboratory for phage typing. In early February, 1978, a female infant was seen

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at the Pediatric Clinic with a pustular rash over the lower abdomen and inner right thigh. Bacteriological examination of the pustular exudate was positive for *S. aureus*. The infant was treated with oral dicloxacillin with good clinical response. This infant had had an uncomplicated nursery stay (22 to 28 January) without apparent cord colonization with *S. aureus*. Phage typing of the isolate—with the currently accepted international set of typing phages 3A, 3C, 6, 29, 42E, 47, 52, 52A, 53, 54, 55, 71, 75, 77, 79, 80, 81, 83A, 84, 85, 94, 95, and 96 at routine test dilution (RTD) and 100× RTD (2)—showed no lytic reaction at RTD, and only a weak 3A[±]/3C⁺ lytic reaction at 100× RTD. A review of phage typing results of *S. aureus* isolates cultured from newborns during January and February did not indicate any similar phage group II patterns.

The occurrence of this weakly reactive group II phage type was not documented again until 27 March, when an infant, born by repeat caesarean section on 23 March, developed erythematous, crusted lesions on the thigh. *S. aureus* was recovered from these lesions and blood cultures taken shortly after delivery; both isolates were characterized by weak lytic reactions with typing phage 3C. During the period February to August, a total of eight infants, including the index case, developed infections (seven involving skin lesions and one involving eye infection) attributable to the group II phage type, whereas three infants were colonized but not infected (Table 1). Four of these infections and one apparent colonization without infection were clustered during an 8-week period, between 23 March and 15 May, with no more than 1 week separating infections. Infected infants often had overlapping nursery stays which would account, in part, for the maintenance and endemicity of the group II strain. It is not unlikely that one or more medical staff personnel may have been colonized with this strain and served as a reservoir for dissemination as well.

The observation that group II staphylococci are frequently associated with skin infections in infants has been well documented (1, 3, 4). The clinical severity of these infections can range from a localized bullous impetigo to a more widespread involvement with or without exfoliation. The value of weak typing reactions in evaluating strains which exhibit apparently dissimilar strong reactions has been described (2). The strains encountered at the Keesler Medical Center were unusual, in that, with one exception, strongly reacting group II strains were never associated with endemic infection or colonization during this period. However, the group II strains recovered consistently produced weak

(± to +) reactions when tested at 100× RTD. It should be noted that propagating strain controls were included with each series of strains for phage typing to determine whether the RTD was satisfactory for use. Additionally, phage stocks used for the preparation of test dilutions (RTD) were as received from the Center for Disease Control, Atlanta, Ga., and had not been further propagated in our laboratory. A review of phage typing reactions of strains submitted from more than 35 Air Force hospitals in the United States indicated that the strain had been encountered only occasionally. Fortunately, during the episode reported here, infections were localized for the most part, and all infants responded readily to treatment with intravenous

TABLE 2. Occurrence of other *S. aureus* phage types among newborn infants, U.S. Air Force Medical Center, Keesler Air Force Base, Miss. (January to August 1978)

Patient	Specimen source	Culture date	Phage type	
			RTD	100 × RTD
1	Nose	3 January	6/47/54/75	6/42E/47/53/54/75
2	Nose	13 January	NT ^a	
3	Eye drainage	16 January	29/52/52A/80/55/71	
3	Cord	16 January	29/52/80/55/71	NT
4	Cord	16 January	NT	
5	Nose	23 January	96	
6	Nose	23 January	54/75	NT
7	Nose	23 January	54/75	
8	Nose	23 January	47/54/75	
9	Nose	30 January	96	NT
10	Nose	30 January	NT	
11	Nose	23 March	NT	
12	Nose	3 April	6/47/75/83A	NT
13	Nose	10 April	94/96	
14	Nose	10 April	NT	
15	Unknown	10 April	NT	55
16	Unknown	10 April	95	NT
17	Cord	24 April	NT	
18	Cord	24 April	NT	
18	Nose	24 April	NT	NT
19	Wound drainage	24 April	95	
20	Cord	24 April	6/47/75/83A	
21	Nose	11 May	NT	NT
22	Nose	22 May	96	
23	Nose	22 May	96	
24	Nose	6 June	NT	NT
25	Nose	18 July	NT	NT
26	Nose	31 July	NT	NT
27	Wound	31 July	47/54/85	NT
28	Nose	31 July	NT	
29	Nose	17 August	47/54/75	
30	Unknown	17 August	47/54/75	NT
31	Nose	28 August	NT	
32	Nose	28 August	NT	
33	Unknown	28 August	NT	NT
34	Nose	28 August	47/54/75	

^a NT, Nontypable.

methicillin, usually followed by a 10-day regimen of oral dicloxacillin. No deaths were recorded. Although no new control measures were instituted during the period of these infections, increased bacteriological monitoring of *S. aureus* colonization among newborn infants was followed, and hand washing by personnel between each infant contact was reinforced. Other *S. aureus* phage types, isolated from newborn infants, were not related to the group II strains (Table 2). There was no unusual increase in the *S. aureus* colonization rate throughout this time.

This experience emphasizes the importance that *S. aureus* infections in the newborn nursery should be monitored, with special emphasis placed on group II strains such as the one de-

scribed. The laboratory should make available comprehensive phage typing results recording strong and weak lytic reactions for all strains found nontypable at RTD.

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Soft-Tissue Infection Due to *Mycobacterium smegmatis*: Report of Two Cases

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Mycobacterium smegmatis is an uncommon pathogen in humans. Fourteen cases of skin or soft-tissue infection due to *M. smegmatis* have been previously reported. We report two cases of posttraumatic *M. smegmatis* infection of the lower extremity. *M. smegmatis* infection produces chronic cellulitis with fistula formation that is most commonly a result of direct traumatic inoculation of contaminated material. Extensive surgical debridement followed by skin grafting has been necessary for cure in the majority of cases.

Mycobacterium smegmatis, a rapidly growing mycobacterium, is an uncommon cause of disease in humans. The first well-described case involving a human was that of a patient with *M. smegmatis* pleuropulmonary infection and was reported by Vonmoos et al. [1] in 1986. Since then, the cases of 25 additional patients with *M. smegmatis* infection, 14 of which had skin or soft-tissue infection, have been reported [2-4]. Among the rapidly growing mycobacteria, *M. smegmatis* is now considered second only to the *Mycobacterium fortuitum* complex as a cause of human disease [5].

Case Reports

Patient 1. A 21-year-old man was involved in an accident during which he was ejected from a motor vehicle and sustained closed fractures of the left scapula and the right first rib as well as a small puncture wound of the distal left leg. The puncture wound was irrigated and cleaned of debris on the day of injury. Six weeks later the patient presented to the orthopedic clinic at our facility (Naval Hospital, San Diego) with complaints of draining of the left-leg lesion of 4 weeks' duration and painful left inguinal lymphadenopathy of 3 days' duration. The patient denied experiencing fever, chills, night sweats, or weight loss.

Physical examination on admission revealed a temperature of 36.8°C and a normal pulse and blood pressure. A warm, erythematous area that measured 9 × 9 cm was present on the lateral aspect of the distal left leg and ankle.

Within this area was a fluctuant mass (1.5 × 2 cm) with central ulceration and seropurulent drainage. Tender, non-fluctuant left inguinal lymphadenopathy with lymphangitic streaking was also present. Radiographs of the distal left tibia revealed periosteal elevation, and a radionuclide bone scan showed intense focal uptake in the medial portion of the left tibia and in the surrounding soft tissue.

The patient underwent operative debridement, and extensive soft-tissue and periosteal necrosis was noted. Gram staining of the debrided tissue revealed many polymorphonuclear leukocytes but no organisms. Ziehl-Neelsen staining was not performed. The patient's wound healed normally, and on the 9th postoperative day acid-fast bacilli were noted in cultures of specimens obtained during the operative debridement. Therapy with cefoxitin (2 g iv every 8 hours) and amikacin (640 mg iv every 12 hours) was begun empirically. Disk diffusion testing of the bacilli showed marked sensitivity to trimethoprim-sulfamethoxazole (TMP-SMZ); consequently, the antibiotic regimen was changed to 1 tablet of TMP-SMZ three times a day. A follow-up visit at 8 weeks showed resolution of the cellulitis with a healed surgical wound, and administration of antibiotics was discontinued. Clinical resolution of the tibial osteomyelitis was also noted, and the patient was still well 7 months after discontinuation of antibiotic therapy. The cultured organism was later identified as *M. smegmatis*.

Patient 2. A 29-year-old severely obese woman was involved in a motor vehicle accident and sustained an avulsion injury (25 × 30 cm) of the anterior left thigh, an open laceration of the right prepatellar bursa, and a traumatic left knee arthrotomy. She was treated at a local hospital, where large amounts of sand and gravel were removed from the wound at initial debridement. Administration of ampicillin, gentamicin, and clindamycin was started empirically. Three days later the patient was transferred to our facility; upon arrival she was febrile (temperature, 38.8°C), tachycardiac (heart rate, 118), and hypotensive (blood pressure, 88/52 mm Hg). Initial examination revealed that the large avulsion injury contained small areas of necrotic tissue and foreign debris; debridement was repeated. *Pseudomonas aeruginosa* grew in cultures of the intraoperative specimens, and antibiotic ther-

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apy was changed to administration of ticarcillin/clavulanate (3.1 g iv every 4 hours). Thereafter, the wounded area progressively improved. Split-thickness grafting of skin was performed 18 days after the third debridement, and the patient was discharged 2 weeks later.

One month after discharge the patient developed cellulitis of the posterolateral aspect of her left thigh, which was empirically treated with dicloxacillin (500 mg po four times a day). Painful, fluctuant nodules developed within the area of cellulitis, and open drainage was performed. Gram staining revealed many polymorphonuclear leukocytes but no organisms. Routine cultures were negative. Acid-fast staining of the debrided tissue was not performed. After 4 months of persistent cellulitis, the patient was readmitted for further evaluation. Examination revealed an erythematous, warm, brawny area of cellulitis (15 × 25 cm) in the left popliteal fossa and posterolateral thigh. Multiple well-formed sinus tracts draining purulent material were present. A radionuclide bone scan and an indium-labeled leukocyte scan showed intense uptake in the medial femoral condyle that was suggestive of osteomyelitis, but a magnetic resonance imaging scan revealed no abnormalities. Ultrasonography revealed an abscess cavity measuring 2.4 × 2 cm within the area of cellulitis, which yielded 8 mL of purulent fluid on needle aspiration. Gram staining of the fluid showed many polymorphonuclear leukocytes with several beaded gram-positive bacilli. Modified Kinyoun carbolfuchsin staining was strongly positive for acid-fast bacilli. The organism was presumptively identified as a *Nocardia* species; the patient was discharged and continued therapy with TMP-SMZ (1 tablet three times a day).

One month later, the patient continued to have pain in and drainage from the thigh lesion. The organism originally isolated had meanwhile been identified as *M. smegmatis*. Antibiotic therapy was changed to the administration of doxycycline (100 mg po twice a day) and ciprofloxacin (750 mg po twice a day) on the basis of sensitivities demonstrated by disk diffusion. The patient continued to have seropurulent drainage from the sinus tracts and required surgical debridement. Extensive necrosis of the subcutaneous tissue that extended to the muscular fascia was noted during surgery. At the time of this writing, the patient was receiving maintenance therapy with ciprofloxacin and doxycycline and was being observed for clinical evidence of recurrent infection.

Discussion

M. smegmatis was isolated initially from syphilitic chancres in 1884 by Lustgarten [6] and, 1 year later, from normal human genital secretions (smegma) by Alvarez and Tavel [7]. This organism has subsequently been recognized as an environmental saprophyte; Tsukamura [8] identified *M. smegmatis* in 25 (38%) of 66 Japanese soil samples. It is interesting that *M. smegmatis* has not been isolated from ei-

ther normal or pathogenic human genital secretions since its initial isolation by Alvarez and Tavel in 1885 [2].

M. smegmatis, a rapidly growing mycobacterium, is microbiologically similar to *M. fortuitum* in that it grows on MacConkey medium without crystal violet, reduces nitrate, and demonstrates iron uptake. However, it can be differentiated from *M. fortuitum* by its growth at 45°C, the negativity of a 3-day test for arylsulfatase, and the delayed formation of pigment at 2 weeks (in 50% of isolates).

As late as 1979, *M. smegmatis* was felt to be nonpathogenic in humans [9]. The first human infection was reported in 1986 by Vonmoos et al. [1], who described a patient with *M. smegmatis* pleuropulmonary infection. The first report of cutaneous disease was published in 1988 by Wallace et al. [2], who reported that 22 clinical isolates had been referred to their lab over a 7-year period. Eleven of these isolates were from patients with cutaneous or soft-tissue infection. A brief description of the patient's medical history accompanied each isolate, but details on the clinical course or response to therapy were not provided.

Plaus and Hermann [3] subsequently reported two cases of soft-tissue infection due to *M. smegmatis* in 1991. Both patients abused anabolic steroids and developed anterior thigh abscesses at the injection site after using a common bottle of veterinary-grade stanozolol that was contaminated with *M. smegmatis*. The patients were treated with erythromycin and amikacin for 2 weeks and then with ciprofloxacin for an additional 6 weeks, but their conditions failed to improve; aggressive debridement followed by skin grafting was required. This procedure resulted in apparent eradication of the infection, as both patients were clinically disease-free 6 months after debridement.

An additional case of cutaneous infection was reported by Roger et al. [4] in 1991. This patient sustained a puncture injury to the ankle while gardening and developed extensive cellulitis with suppuration. The patient was treated with various antibiotic combinations to which the isolate was sensitive in vitro, but failed to respond and required aggressive surgical debridement with subsequent skin grafting.

In summary, *M. smegmatis* is a rare but clinically significant environmental pathogen and should be considered in the diagnosis of chronic cutaneous or soft-tissue infection. A history of a soil-contaminated wound should raise the clinical suspicion of infection with this pathogen. Extensive surgical debridement followed by skin grafting has been necessary for the eradication of infection for most patients. Adjunctive antibiotic therapy should be considered and can be chosen on the basis of in vitro sensitivity data [10].

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Characterization of Hemolysin in Extracellular Products of *Pseudomonas cepacia*

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Pseudomonas cepacia is recognized as an opportunistic pathogen in immunocompromised patients. We screened 120 strains of *P. cepacia* isolated from clinical specimens for production of extracellular products. About 70% of these strains produced lipase, protease, and lecithinase, but only 4% produced hemolysin. A hemolysin produced by *P. cepacia* JN106 was characterized. The hemolysin was most active against human erythrocytes. Horse, sheep, chicken, and rabbit erythrocytes were also susceptible. The hemolysin was heat labile and was inhibited by sterols but was not activated by 2-mercaptoethanol and dithiothreitol. Four hemolysin-negative mutants obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment produced the other extracellular products. A 58-kilobase-pair plasmid found in the parent strain was also found in the mutant strains, suggesting that the hemolysin gene resides on the chromosome.

In the genus *Pseudomonas*, *Pseudomonas cepacia* has the most versatile ability to catabolize various organic compounds (24). It is highly resistant to many antibiotics and disinfectants and has been isolated with increasing frequency from clinical specimens and hospital environments (3, 6, 20, 27). The species is recognized as an opportunistic pathogen and is associated with various types of nosocomial infections (4, 7, 22, 27). In addition, *P. cepacia* now complicates cystic fibrosis (13).

There are few reports on the virulence factors of *P. cepacia*. McKeivitt and Woods (19) have reported the production of virulence factors by 48 strains of *P. cepacia* isolated from patients with cystic fibrosis. A majority of strains produced protease and lipase, and about one-half of the strains produced smooth lipopolysaccharide. On the other hand, none of the strains produced elastase, cytotoxins, or ADP-phosphoribosyl transferase.

This study was initiated to determine the virulence factors of *P. cepacia*. One hundred and twenty strains isolated from clinical specimens were screened for extracellular products. In addition, a hemolysin produced by one of the strains was characterized.

MATERIALS AND METHODS

Bacterial strains. One hundred and four strains of *P. cepacia* isolated from clinical specimens in Juntendo University Hospital, Tokyo, Japan, from 1983 to 1984 were generously provided by T. Oguri and J. Igari. Among 79 strains of known origin, 15 strains were isolated from sputum, 12 strains from pus, 11 strains from urine, and 9 strains from cerebrospinal fluid; and 4 strains each were from blood, catheters, and drain tubes. Other strains were provided by S. Oiyé, Yamaguchi University Hospital, Ube, Yamaguchi, Japan, and E. Yabuuchi, Gifu University, Gifu, Japan. Identification of strains was confirmed by biochemical tests (9). Strains were stored in sterile glycerol solution (10% [wt/vol]) at -80°C.

Detection of extracellular products. Protease (28), elastase (23), and lecithinase (8) were determined by plate assays;

lipase production was determined by Tween 80 hydrolysis (26); and hemolysin production was determined by using plates containing 7.5% defibrinated sheep blood in heart infusion agar (Eiken Co. Ltd., Tokyo, Japan). Results were determined after incubation at 28°C for 48 h.

Preparation of crude hemolysin. Cells were grown in nutrient broth (Difco Laboratories, Detroit, Mich.) or dialysate of brain heart infusion broth (Eiken Co.) for about 48 h at 28°C with shaking. When the A_{660} of the culture reached 1.5, the culture supernatant was obtained by centrifugation (9,000 × *g*, 10 min). Crude hemolysin was prepared by ammonium sulfate fractionation and by taking the fraction between 20 and 60% saturation. This fraction was then dialyzed against 10 mM Tris hydrochloride (pH 7.4). The preparations of culture supernatant and crude hemolysin were stored at -80°C until use. Protein concentrations were determined by the method described by Lowry et al. (18).

Hemolysin assay. The hemolysin assay mixture contained 10 mM Tris hydrochloride (pH 7.4)-160 mM NaCl (hemolysin assay buffer), 2% suspensions of sheep erythrocytes that had been washed with saline, and an appropriate volume of sample containing hemolysin in a total volume of 2 ml. Control experiments for spontaneous lysis or complete lysis were carried out without hemolysin and with 0.2% sodium dodecyl sulfate, respectively. Reaction mixtures were incubated at 37°C for 10 min and chilled on ice for 2 min to stop the reaction. The unlysed erythrocytes were removed by centrifugation at 1,500 × *g* for 2 min, and the A_{530} of the supernatant was determined. The activity resulting in 50% hemolysis of 2 ml of 2% sheep erythrocyte suspensions was defined as 1 hemolytic unit (HU).

Studies on effects of various reagents on hemolysin. The culture supernatant of strain JN106 (8 HU/ml) was preincubated at 0°C for 2 h with *N*-ethylmaleimide (1 mM), *p*-chloromercuribenzoate (1 mM), 2-mercaptoethanol (1 mM), dithiothreitol (1 mM), or disodium EDTA (1 mM and 10 mM); and the residual activity was determined. The effect of CaCl₂ on hemolysin was determined by the addition of 1 or 10 mM CaCl₂ to the assay mixture. Sterols were dissolved in ethanol to make a 5 mM solution which was serially diluted with hemolysin assay buffer just before use. A small volume of crude hemolysin was added to 1 ml of sterol solution and

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TABLE 1. Extracellular products produced by 120 clinical isolates of *P. cepacia*

Extracellular product ^a	No. of positive strains (%)
Protease	83 (69)
Lecithinase	80 (67)
Lipase	88 (73)
Hemolysin	5 (4)
Elastase	0 (0)

^a Extracellular products were determined by plate assays, as described in the text.

incubated at 0°C for 30 min. Then, the hemolysin activity was determined under standard assay conditions.

Mutagenesis. An overnight culture (4 ml) of *P. cepacia* JN106 was incubated with 12 ml of fresh nutrient broth at 28°C for 4 h with shaking. Cells were harvested by centrifugation, washed once with 0.1 M citrate buffer (pH 5.0), and suspended in 8 ml of the same buffer. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; 2 ml) was added to a final concentration of 1 mg/ml, followed by incubation at 28°C for 90 min without shaking. To screen hemolysin-negative mutants, cells were washed twice with carbon source-free M9 medium (5) and spread on sheep blood-agar plates, which were incubated at 28°C. About 0.5% of the cells remained viable after NTG treatment.

Isolation of plasmid DNA and digestion with restriction enzyme. Plasmid DNA was isolated by the method of Kado and Liu (15). DNA was subjected to 0.7% agarose gel electrophoresis in Tris-borate buffer (89 mM Tris, 2.5 mM EDTA, 89 mM boric acid [pH 8.0]) at a constant 100 V. Gels were stained with ethidium bromide (1 µg/ml) and photographed. Digestion of plasmid DNA with *Bam*HI (Takara Shuzo Co. Ltd., Kyoto, Japan) was performed by the method of the supplier.

RESULTS

Detection of extracellular products. One hundred and twenty clinical isolates of *P. cepacia* were screened for the production of protease, lecithinase, lipase, hemolysin, and elastase (Table 1). The majority of strains produced protease, lecithinase, and lipase. Only 5 strains produced hemolysin, as indicated by a clear zone of hemolysis around the colonies on sheep blood-agar plates. All the hemolysin-producing strains also produced protease, lecithinase, and lipase. None of the strains produced elastase. Among 120 strains, 30 strains did not produce any of the extracellular products described above.

Characterization of hemolysin produced by *P. cepacia* JN106. Because hemolysins produced by various bacteria are known to be involved in bacterial virulence, we characterized the *P. cepacia* hemolysin produced by strain JN106.

TABLE 2. Sensitivity of erythrocytes from various animal species to strain JN106 hemolysin^a

Source of erythrocytes	Relative sensitivity (%)
Human	195
Horse	142
Sheep	100
Chicken	97
Rabbit	77

^a Culture supernatant (8.8 HU/ml) was used.

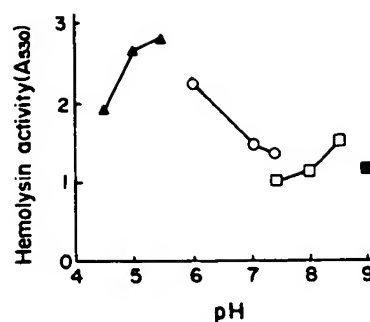


FIG. 1. Effect of pH on hemolysin activity. The culture supernatant of *P. cepacia* JN106 (8.0 HU/ml) was incubated with 2% sheep erythrocyte suspensions in 10 mM buffers of various pHs containing 160 mM NaCl. Symbols: ▲, sodium acetate buffer; ○, sodium phosphate buffer; □, Tris hydrochloride buffer; ■, glycine-NaOH buffer.

When the cells of JN106 were grown in nutrient broth or in dialysate of brain heart infusion broth at 28°C for 48 h without shaking, the culture supernatant contained approximately 8 HU of hemolysin per ml. On the other hand, the activity could not be detected when the cells were grown with shaking but, otherwise, under the same conditions.

About 60% of the hemolysin activity in the culture supernatant was recovered by ammonium sulfate fractionation in the fraction from 20 to 60% saturation. Attempts to purify the hemolysin by gel chromatography or ion-exchange column chromatography were hindered by aggregation of the hemolysin (data not shown).

For the standard assay of hemolysin, sheep erythrocytes were used. In addition to sheep erythrocytes, the hemolysin showed cytolytic activity against human, horse, chicken, and rabbit erythrocytes (Table 2). Human erythrocytes were twofold more sensitive to the hemolysin than were sheep erythrocytes.

The effect of pH on hemolysin activity was determined with the culture supernatant, which showed the highest activity at pH 5.5. This was twice as much as the activity at pH 7.4 (Fig. 1).

Inactivation studies. The hemolysin was heat labile, and 94 and 97% of the activity was lost by heat treatment at 56°C for 30 min or 100°C for 5 min, respectively (Table 3).

The activity of hemolysin against sheep erythrocytes was not affected by *N*-ethylmaleimide, *p*-chloromercuribenzoate, 2-mercaptoethanol, dithiothreitol, EDTA, or CaCl₂. In addition, the hemolysin was not activated by 1 mM dithiothreitol, even after incubation at 4°C for 15 h.

Inhibition of hemolysin by sterols. Cholesterol and 7-dehydrocholesterol completely inhibited hemolysin activity at a concentration of 3 µM, and 50% inhibition was observed on the addition of 0.5 µM of the sterols (Fig. 2). In addition, ergosterol, dihydrocholesterol, and stigmasterol showed 50% inhibition at concentrations of 3 to 5 µM. Concentra-

TABLE 3. Heat stability of strain JN106 hemolysin

Treatment ^a	Residual activity (%)
100°C, 5 min	3.2
100°C, 20 min	1.8
56°C, 30 min	5.6

^a A total of 0.1 ml of crude hemolysin (10 HU) was dissolved in hemolysin assay buffer and incubated under various conditions.

tions of sterols to give 50% inhibition were variable, depending on the hemolysin preparations. No inhibition was observed by 50 μ M dehydroepiandrosteron, pregnenolone, or estradiol (data not shown).

Isolation of hemolysin-negative mutants of JN106. *P. cepacia* JN106 produced protease, lecithinase, and lipase, in addition to hemolysin. To establish that the hemolysin activity was not associated with the other extracellular products, hemolysin-negative mutants were isolated after NTG treatment. By screening 1,500 clones, 4 hemolysin-negative mutants were obtained (Table 4). The mutant JN106 did not produce any hemolysin, as judged by plate or tube assay, by using a sample of the 20 to 60% ammonium sulfate fraction containing 0.25 mg of protein. In contrast, the same ammonium sulfate fraction of the parent strain (crude hemolysin) had approximately 40 HU/mg of protein. Other mutants appeared to be slightly leaky, producing a narrow hemolytic zone on plates after incubation for 4 days at 28°C. Other extracellular products were produced normally by the mutants, except for mutant JN1075, which did not produce protease (Table 4). These results indicate that the hemolysin activity of *P. cepacia* JN106 is not associated with the other extracellular products.

Plasmid analysis of JN106. Strain JN106 was found to carry a plasmid of approximately 58 kilobase pairs, based on the size of restriction fragments produced by digestion with *Bam*HI. All the mutant strains also carried a plasmid of the same size as that carried by the parent strain. It was assumed that the plasmid was not involved in the hemolysin synthesis of JN106.

DISCUSSION

The results of the survey of extracellular products of 120 strains of *P. cepacia* described in this report coincided well with those of McKevitt and Woods (19), who screened for extracellular products of 48 strains of *P. cepacia* isolated from patients with cystic fibrosis. It appears, therefore, that the described pattern of extracellular products (Table 1) may represent the pattern of *P. cepacia* populations of clinical origin. Gonzalez and Vidaver (11) reported that onion maceration tests and pectolytic activity at low pHs were positive in strains of plant origin, whereas these activities were negative or minimal in strains of clinical origin.

Hemolysin is considered to be one of the virulence factors of several bacteria such as *Streptococcus pyogenes* (1) *Staphylococcus aureus* (14), *Vibrio parahaemolyticus* (21),

TABLE 4. Properties of hemolysin-negative mutants of strain JN106

Strain	Production of ^a :			
	Hemolysin	Protease	Lecithinase	Lipase
JN106 (wild type)	+	+	+	+
JN1066	\pm^b	+	+	+
JN1067	—	+	+	+
JN1070	\pm	+	+	+
JN1075	\pm	—	+	+

^a Extracellular products were determined as described in the text.

^b Slight hemolysis was found after incubation for 4 days.

Vibrio vulnificus (16), *Escherichia coli* (29), and *Pseudomonas aeruginosa* (17). The heat-labile hemolysin produced by *P. aeruginosa* is phospholipase C, which catalyzes the hydrolysis of phosphatidylcholine (lecithin) to phosphocholine and diacylglycerol (2). The hemolysin-producing strain *P. cepacia* JN106 also produced lecithinase, as determined by the egg yolk reaction. Association of the hemolytic activity with lecithinase is unlikely, because hemolysin-negative mutants still produced lecithinase.

The hemolysin produced by strain JN106 was inhibited by several sterols. Cholesterol and 7-dehydrocholesterol showed the strongest inhibition of the hemolysin. The inhibition pattern of *P. cepacia* hemolysin by sterols was very similar to that of streptolysin O, a representative of thiol-activated and cholesterol-binding cytolytins (25). In contrast to streptolysin O, however, the *P. cepacia* hemolysin was not activated by 2-mercaptoethanol or dithiothreitol. Hemolysin produced by *V. vulnificus* is also not affected by 2-mercaptoethanol or dithiothreitol, although it is inhibited by cholesterol (12).

Results of many studies have shown that the genetic determinants for certain virulence factors are carried by plasmids. For example, *E. coli* strains of animal origin carry a hemolysin gene on a plasmid (10). We analyzed strain JN106 and found that it contains a 58-kilobase-pair plasmid. The hemolysin determinant, however, is unlikely to reside on the plasmid, because hemolysin-negative mutants still carry the plasmid.

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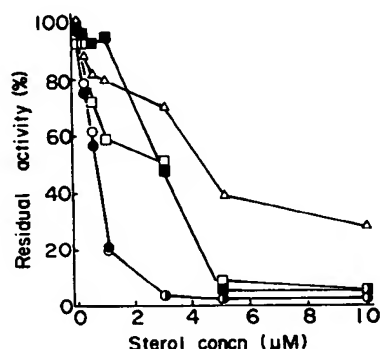


FIG. 2. Effect of sterols on *P. cepacia* hemolysin. Crude hemolysin (1 HU) was incubated with various concentrations of sterols. Symbols: ○, cholesterol; ●, 7-dehydrocholesterol; □, dihydrocholesterol; ■, ergosterol; △, stigmasterol.

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MiniReview

Virulence factors of *Burkholderia cepacia*

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Introduction

Originally named following its identification as a cause of soft rot in onions, *Burkholderia cepacia* has also been known as *Pseudomonas multivorans* and *Pseudomonas kingae* [1,2], and until most recently, as *Pseudomonas cepacia* [3]. A proposal for the transfer of 7 species of the genus *Pseudomonas* RNA Homology group II to a new genus *Burkholderia* with the type-species *Burkholderia cepacia* has been validated [3]. For the purpose of this review the name *B. cepacia* will be used.

Once considered solely as a phytopathogen, *B. cepacia* is now recognized as an important pathogen in nosocomial infection and in patients with chronic granulomatous disease and particularly in those with cystic fibrosis (CF). CF is the most common autosomal recessive lethal disease in Caucasian populations with an incidence of approximately 1 in 2500 live births and a carrier

frequency of 1 in 20. The basic cause of the pathophysiological symptoms of CF is a defect in epithelial ion transport which results in viscous dehydrated bronchopulmonary and gastrointestinal secretions. Build-up of viscid mucus is associated with impaired mucociliary clearance and susceptibility to bacterial colonization which in turn initiates a vicious cycle of chronic inflammatory reaction. The susceptibility of CF patients to pulmonary colonization has been recognised since the earliest descriptions of the disease when patients seldom survived infancy. Advances in management of CF have meant that today most patients survive to early adulthood. However, this increased longevity has in part created its own problems including the emergence of new opportunistic pathogens, including and most notably, *B. cepacia*.

The last decade has seen *B. cepacia* emerge as a particular problem amongst patients with CF, where colonization may be symptomless or associated with a slow decline in lung function. A more serious clinical outcome, not observed with other CF pathogens, in which some colonized CF patients experience *B. cepacia* bacteraemia and/

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or succumb to an accelerated and fatal deterioration in pulmonary function [4-7], is central to the current concern over *B. cepacia* in the CF community.

Based on nucleic acid homology, *B. cepacia* is more closely related to *B. pseudomallei*, *B. mallei* and *B. gladioli* than to *P. aeruginosa* and other fluorescent pseudomonads, and was placed in the separate subgroup, *Pseudomonas* RNA homology group II. *B. cepacia* is nutritionally versatile, with minimal growth requirements and the ability to survive in unfavourable environments: it has been isolated from disinfectants and antiseptics and can even use penicillin G as a nutrient [8-11]. The organism is intrinsically resistant to most antibiotics, and even if individual strains show in vitro susceptibility to an antibiotic, there is little clinical response [10,12-15].

In contrast to the large amount of information on *P. aeruginosa* virulence factors, knowledge of the virulence factors and pathogenesis of *B. cepacia* is scanty. Animal models have indicated that *B. cepacia* is less virulent than *P. aeruginosa* [16]. The aim of this review is to discuss the main features and properties of *B. cepacia* and, in particular, to focus on those which may contribute to its ability to colonize patients with CF.

Colonization and adherence

The ability of a potential pathogen to adhere to the host mucosal or epithelial cell surfaces is often pivotal in the subsequent establishment of infection. Few potential adhesins have been described for *B. cepacia*, and most attention to date has been focused on the adhesive properties of fimbriae.

Electron microscope studies have shown that approximately 60% of *B. cepacia* strains express peritrichous fimbriae (see Fig. 1) [17,58]. Other *B. cepacia* strains possess polar fimbriae, similar to those expressed by *B. aeruginosa* [18]. Kuehn et al. [17] showed that outer membrane protein preparations of *B. cepacia* were enriched with 3 proteins (16, 20 and 40 kDa) which were not present in a non-fimbriated strain. The fimbrial subunit was identified as the 16 kDa protein; the

protein appeared similar to those seen in other bacteria and showed homology with PAK fimbriae of *P. aeruginosa* [17]. This data contrasts with that of Saiman et al. who found minimal cross-reactivity with anti-*P. aeruginosa* anti-pilin monoclonal antibodies and no homology between *P. aeruginosa* pilin gene probes and *B. cepacia* genomic DNA [18,19]. It is possible that sequence variation exists among the pilin genes of different *B. cepacia* strains and that any individual pilin gene probe from *P. aeruginosa* may not reveal a specific *B. cepacia* gene [17].

The presence of fimbriae increases the ability of *B. cepacia* to adhere to pneumocytes in vitro [17]. In vitro binding experiments by Kirvan et al. [20] demonstrated that both *B. cepacia* and *P. aeruginosa* adhere to the same Gal β 1-4GalNAc sequence present in many asialoglycolipids. The experiments of Saiman et al. [19] did not demonstrate competition for epithelial receptors, indicating that different epithelial receptors may be used preferentially by each of the *Pseudomonas* species or that the bacteria may bind to each other. Binding of 2 *B. cepacia* strains to epithelial monolayers increased in the presence of *P. aeruginosa* indicating a possible synergistic relationship whereby *P. aeruginosa* exo-products modify epithelial cell surfaces, exposing receptors and facilitating increased *B. cepacia* attachment [19]. It must be stressed, however, that not all CF patients are colonized with *P. aeruginosa* prior to acquisition of *B. cepacia*: in the Edinburgh CF clinic 38% of patients with *B. cepacia* are not co-colonized with *P. aeruginosa* [6].

Sajjan et al. [21] were able to demonstrate specific binding of *B. cepacia* isolates from patients with CF to both CF and non-CF mucins as well as to buccal epithelial cells. Unfortunately no typing data was available to exclude the possibility of clonal relationships between the strains [21]. The degree of binding observed with *B. cepacia* is considerably less than that observed with *P. aeruginosa* [58]. Deglycosylation of mucin indicated that the mucin receptors for *B. cepacia* include N-acetylglucosamine and N-acetylgalactosamine. Isolates exhibiting the highest mucin binding values tended to correlate with those patients with severe illness leading to speculation

that variability in the binding of different *B. cepacia* isolates to respiratory mucin may contribute to morbidity and mortality, and may explain why some *B. cepacia* strains colonize patients transiently whereas other strains, once acquired, are never lost. A sparsely distributed 22 kDa pilin-associated protein was identified as a mucin binding adhesin specific to piliated strains of *B. cepacia* [22].

Siderophores

Production of siderophores enables bacteria to compete for iron with host iron-binding proteins including transferrin and lactoferrin and has been correlated with the ability of various bacteria to

establish and maintain infection. *B. cepacia* strains express at least 3 siderophore-mediated iron transport systems, including pyochelin, cepabactin and azurechelin [23–26]. Pyochelin produced by *B. cepacia* is chemically unrelated to the pyochelin siderophore of *P. aeruginosa* [23,24]. A 14 kDa ferripyochelin binding protein, present in increased amounts in the outer membrane of iron-starved *B. cepacia* cells, has also been described [23]. Morbidity and mortality in infected CF patients has been correlated to the production of pyochelin. Although such evidence may point to a role in pathogenicity, half of the clinical isolates investigated by Sokol [23] were pyochelin-negative. Pyochelin may increase the ability of *B. cepacia* to disseminate throughout the lungs and perhaps induce a greater inflammatory



Fig. 1. *Pseudomonas cepacia* J1359 in the process of dividing into 2 separate cells which exhibit peritrichous pili. Staining is 2% w/v phosphotungstic acid. Magnification $\times 25000$.

response due to the increased area of infection in the lung [27]. Exogenously supplied pyochelin enhanced the virulence of non-pyochelin producing *B. cepacia* strains in a chronic pulmonary model in rats [27]. Meyer et al. [26] showed that *B. cepacia* ATCC25416 excreted both pyochelin and a lower molecular mass compound, cepabactin, which strongly chelated Fe III and facilitated iron translocation. Azurechelin, another distinct iron-binding compound, has been identified in 88% of *B. cepacia* strains isolated from the respiratory tract [25].

Extracellular virulence factors

B. cepacia produces a number of extracellular products including protease, gelatinase, haemolysin and lipase, although no pathogenic role for these factors has been demonstrated [28,29]. Unlike *P. aeruginosa*, *B. cepacia* does not appear to produce toxin A, exoenzyme S, or other detectable extracellular factors capable of producing a cytotoxic effect in vitro [28]. In a study of putative pathogenic factors of *B. cepacia* [30] a number of characteristics were demonstrated more frequently in isolates from CF patients than control isolates. These factors included production of catalase, ornithine decarboxylase, valine aminopeptidase, C14 lipase, alginate, and trypsin; reduction of nitrate to nitrite; hydrolysis of urea and xanthine and complete haemolysis on bovine red blood cells. The role of any of these factors in respiratory colonization or infection in CF patients is not clear [30]. Indeed, an epidemic strain of *B. cepacia* CF5610 associated with fatal clinical outcome in CF does not produce C14 lipase or haemolysis [6].

Molecular studies of the *Pseudomonas* exotoxin A gene by Vasil et al. [31] concluded that the production of exotoxin A and the presence of the exotoxin A gene are probably limited to *P. aeruginosa* and is not found in other *Pseudomonas* spp. Southern hybridization experiments under low, medium and high stringency conditions with an exotoxin A gene probe failed to produce a positive signal with any of 8 *B. cepacia* strains tested. Similar experiments con-

ducted by ourselves also failed to demonstrate the presence of the exotoxin A gene in 3 environmental strains of *B. cepacia* although a positive band was obtained with *B. cepacia* CF5610 strain isolated from a patient with CF. However, growth of this strain in both iron replete and depleted medium and subsequent analysis of the cell free culture supernate by polyacrylamide gel electrophoresis and immunoblot analysis with anti-*P. aeruginosa* exotoxin A antisera, failed to confirm production of a 66 kDa protein equivalent to *P. aeruginosa* toxin A. (J.W. Nelson, unpublished results).

The extracellular proteinase of *B. cepacia*, a 34 kDa protein, has antigenic similarities to *P. aeruginosa* elastase and cleaves gelatin, hide powder, collagen but not human immunoglobulin IgG, IgM, secretory IgA, or IgA [32]. Intratracheal instillation of purified proteinase into rat lungs produces a bronchopneumonia characterized by polymorphonuclear cell infiltration and proteinaceous exudation into large airways. Active immunization of rats with *B. cepacia* proteinase elicits an immunological response although this is not protective against subsequent lung infection with *B. cepacia* [32].

There is also evidence that lipases, particularly phospholipases, may play an important role in bacterial virulence [33,34]. Phospholipase C is an enzyme that cleaves phosphatidylcholine, a major lung surfactant, to yield phosphorylcholine and diacylglycerol, and has been associated with cytopathology of lung tissue. *B. cepacia* has frequently been described as being lipolytic [28,33-35]. McKevitt and Woods [28] reported that 32 of 48 strains of *B. cepacia* isolated from CF patients demonstrated lipase activity on egg-yolk agar whilst Carson et al. [35] showed that *B. cepacia* could hydrolyse Tween 20, 40, and 80. In another study [34] 6 out of 10 clinical strains of *B. cepacia* from the sputum of CF patients produced lecithinase by the egg-yolk reaction, whilst lipase activity on 4 different Tweens was strain-dependent. Purified enzyme had a molecular weight of 25 000 and was not cytotoxic for Hela cells or for mice injected intravenously with purified lipase. It has been reported, however, that lipase adversely affected the phagocytic function of rat pulmonary

alveolar macrophages in a dose-dependent manner [36]. Phagocytosis of *B. cepacia* by rat pulmonary alveolar macrophages was significantly reduced when the cells were either preincubated with lipase or when phagocytosis occurred in the presence of the lipase [36]. Scanning electron microscopy showed that the macrophages exposed to *B. cepacia* lipase had fewer pseudopodia, microvilli and other projections compared to untreated macrophages. Thus *B. cepacia* lipase may be an important virulence factor which allows the bacteria to evade the mammalian host defence system.

B. cepacia produces a heat-labile haemolysin which has both phospholipase C and sphingomyelinase activities [37]. Haemolytic and phospholipase C (lecithinase) expression in *B. cepacia* appears to be a complex phenomena. The study of Nakazawa et al. [29] found that only 4% of clinical isolates were β -haemolytic, whilst 67% of isolates produced lecithinase. Others have found higher percentages of haemolytic isolates of *B. cepacia* if a variety of erythrocyte types were tested, including a study of clinical isolates of *B. cepacia* from CF patients which found that 40% were haemolytic when erythrocytes from various animals were tested [37]. Unlike the PLC activity of *P. aeruginosa* the PLC activity in *B. cepacia* does not correlate with haemolytic activity [29,37]. However, all haemolytic strains produce detectable lecithinase activity, and strains of *B. cepacia*, whether haemolytic or non-haemolytic, appear to produce detectable amounts of extracellular PLC activity. In contrast to the consistent patterns observed in the PLC gene of *P. aeruginosa* there is hypervariability in genetic organization of the PLC gene of *B. cepacia* [37]. The variable manner in which a *B. cepacia* PLC specific gene probe hybridizes with restricted *B. cepacia* DNA, the variability in expression of haemolytic and PLC activities of different strains, and the association of DNA arrangements with conversion of an Hly + to an Hly - variant may be related to the relatively large number of distinct insertion sequences (IS) reported for *B. cepacia* (> 25) [37,38]: in contrast these elements have yet to be discovered in *P. aeruginosa*. Some of these IS elements of *B. cepacia*, can be found

in multiple copies and have been shown to both activate or inactivate gene expression.

Cell surface antigens

Lipopolysaccharide

B. cepacia strains isolated from patients with CF may express either the rough (R) or smooth (S) lipopolysaccharide (LPS) phenotype, whereas the majority of *B. cepacia* strains isolated from other clinical conditions or from the environment express S-LPS (S.L. Butler, unpublished results). This is in agreement with the study of McKevitt and Woods [28] where 22 strains examined possessed S-LPS and 26 strains possessed R-LPS. The epidemic strain of *B. cepacia* isolated from a number of CF patients in the UK invariably has a R-LPS phenotype and is associated with the appearance of dry colonies [6]. There is no evidence to date to confirm that *B. cepacia* strains undergo a phenotypic change from S to R LPS within the CF lung as is observed with *P. aeruginosa*.

Western blotting and absorption studies demonstrated that a significant proportion of serum antibodies from *B. cepacia*-infected CF patients which reacted with the core LPS of *B. cepacia* did not react with the core LPS of *P. aeruginosa* [39]. These observations indicate differences in the structure and composition of core LPS between *B. cepacia* and *P. aeruginosa* confirming previous findings, including the lack of phosphorus in the core of *B. cepacia* LPS [40] and the inability of a monoclonal antibody reactive with *P. aeruginosa* and *P. fluorescens* core LPS to react with *B. cepacia* [41]. Core heterogeneity between different isolates of *B. cepacia* may also exist because immunoblotting demonstrated that serum from patients colonized with *B. cepacia* produced a band reactive with some but not all core LPS preparations [39].

Initial chemical analysis of *B. cepacia* LPS indicated the absence of detectable 3-deoxy-D-manno-2-octulosonic acid (KDO) in LPS from *B. cepacia* [42,43]. However, Straus et al. [44] reported the isolation of KDO from the culture supernate of 2 out of 10 strains of *B. cepacia* and

in a further study KDO was demonstrated in 6 clinical isolates of *B. cepacia* and all 6 LPS preparations were equally toxic for mice when injected intraperitoneally [45]. Compared with LPS from *P. aeruginosa* that from strains of *B. cepacia* has less phosphorus and more heptose. Glucose and rhamnose were the major saccharide components of LPS from the organisms tested [42]. An extracellular material isolated from a clinical *B. cepacia* consisted of a surface carbohydrate antigen, LPS and protein, the toxicity of which appeared to be associated with the LPS portion of the complex [46]. It has been proposed that this extracellular toxic complex produced by *B. cepacia* is responsible for the lethality and extensive pulmonary tissue necrosis associated with pneumonia produced by this organism.

Outer membrane proteins

B. cepacia produces 5 major outer membrane proteins A (56 kDa), B (38 kDa), C (37 kDa), D (28 kDa) and E (21 kDa). The C and D proteins have been identified as porin proteins [47,48], and appear to be antigenic in most patients with CF who are chronically colonized with *B. cepacia* [48,49]. In the study of Anwar et al. [55], outer membrane protein profiles of magnesium-depleted cells were much simpler than that of iron-depleted cells and nutrient broth grown cells. Synthesis of a 66 kDa outer membrane protein was induced when *B. cepacia* was grown under iron depletion. *B. cepacia* isolates from individual CF patients may exhibit marked phenotypic variability, including manifestation of different patterns of outer membrane proteins separated on a polyacrylamide gel: up to 5 OMP patterns have been identified from *B. cepacia* isolates derived from a single strain [50].

Various studies indicate that the outer membrane of *B. cepacia* is a major contributing factor in the β -lactam resistance of this species, retarding the diffusion of β -lactams to their penicillin-binding protein targets [47,51,52]. Resistance to aminoglycosides and hydrophobic compounds in *B. cepacia* is largely due to the low outer membrane permeability [51]. Loss of the major porin protein D and decreased expression of protein C

may also be associated with high level β -lactam resistance in some CF isolates of *B. cepacia* [52]. Production of β -lactamases, including carbapenemases capable of hydrolyzing the most potent and broad spectrum of the β -lactam antibiotics, imipenem and meropenem, also contribute significantly to the resistance of *B. cepacia* [53].

Exopolysaccharide

Production of alginate by mucoid strains of *P. aeruginosa* is the major virulence determinant associated with strains which colonize the lungs of patients with CF. In contrast, *B. cepacia* does not appear to produce alginate. PCR studies with primers of the *P. aeruginosa algD* gene, encoding the essential enzyme GDP mannose dehydrogenase, indicate that this gene was absent in 10 *B. cepacia* strains studied and therefore that *B. cepacia* is unlikely to produce an alginate-like polymer (J.W. Nelson, unpublished results). Additional studies in our laboratory and by Sage et al. [54] showed that some *B. cepacia* strains do produce an exopolysaccharide comprising galactose, glucose, mannose, glucuronic acid and rhamnose, with lesser amounts of uronic acid: no mannuronic or guluronic acid was detected. Surveys of clinical isolates from patients with CF indicate that there is no correlation between the ability of *B. cepacia* to colonize the respiratory tract and capacity to form exopolysaccharide [6,54]. In contrast, Straus et al. [46] observed that 1 strain of *B. cepacia* produced an alginate-like compound containing 72% guluronic acid with 1.75% acetylation.

Evasion of the immune system

Immunological studies on *B. cepacia* colonization of patients with CF indicate that the organism persists despite a considerable antibody response and suggest the possibility of immune-mediated damage. Aronoff et al. [48,49] demonstrated the presence of IgG antibodies to outer membrane antigens of *B. cepacia* in serum from patients with CF colonized with *B. cepacia* and/

or *P. aeruginosa*. These authors concluded that some *B. cepacia* outer membrane components may be antigenically related to those of *P. aeruginosa* and that colonization with *B. cepacia* occurs in the presence of antibodies specific for the outer membrane of the organism. Serum IgG and sputum IgA antibodies directed towards the core LPS of *B. cepacia* have also been described [39].

Investigations into the bactericidal effect of human serum have shown a large variation in the responses of the *B. cepacia* strains investigated. All strains expressing R-LPS were serum-sensitive under a variety of test conditions whilst strains expressing S-LPS exhibit a range of responses (S.L. Butler, unpublished results). Anwar et al. [55] showed that a *B. cepacia* strain grown in different nutrient depletions in batch culture showed varying degrees of sensitivity to engulfment and killing by human polymorphonuclear leucocytes (PMN) and to killing by human serum. The wide range of sensitivity shown by the organism may reflect the phenotypic variation in cell envelope composition caused by specific nutrient depletions. Patients with chronic granulomatous disease (GCD) are at particular risk of infection with *B. cepacia*, which is able to resist neutrophil-mediated non-oxidative bactericidal killing (D.P. Speert, personal communication). The ability of *B. cepacia* to survive a pronounced humoral response and other immunological defences is intriguing and requires further investigation. Indeed there is a suggestion that *B. cepacia* may invade and survive within respiratory epithelial cells, enabling the organism to persist within the CF lung [56].

Concluding remarks

Acquisition of *B. cepacia* is a major concern among patients with CF although the exact pathophysiological role of the organism remains controversial and unsolved. Epidemiological data and the use of phenotypic and genotypic typing systems for *B. cepacia* suggest that certain strains are particularly transmissible, although there is no evidence at present that some strains are more virulent. The role of any of the described viru-

lence factors of *B. cepacia* relating to its pathogenesis in patients with CF remains unclear. Enhanced adhesion to mucin of certain *B. cepacia* strains may aid initial colonization whilst multi-resistance to antibiotics and possible intracellular localization may contribute to persistence of the organism. Production of anti-*B. cepacia* antibodies by the host and subsequent immune complex mediated damage, is probably responsible for pulmonary decline. The development of a CF mouse model carrying precise and clinically relevant mutations [57] will allow in vivo investigation of *B. cepacia* colonisation and virulence factors. Current concern over *B. cepacia* colonisation amongst CF patients has highlighted the urgent need to identify *B. cepacia* colonising factors and the pathophysiological and/or immunological factors which account for the rapid clinical deterioration in some CF patients.

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The emergence of a highly transmissible lineage of *cbl*⁺ *Pseudomonas* (*Burkholderia*) *cepacia* causing CF centre epidemics in North America and Britain

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The rapid increase in *Pseudomonas* (*Burkholderia*) *cepacia* infection in cystic fibrosis (CF) patients suggests epidemic transmission, but the degree of transmissibility remains controversial as conflicting conclusions have been drawn from studies at different CF centres. This report provides the first DNA sequence-based documentation of a divergent evolutionary lineage of *P. cepacia* associated with CF centre epidemics in North America (Toronto) and Europe (Edinburgh). The involved epidemic clone encoded and expressed novel cable (Cbl) pili that bind to CF mucin. The sequence of the *cblA* pilin subunit gene carried by the epidemic isolates proved to be invariant. Although it remains to be determined how many distinct, highly transmissible lineages exist, our results provide both a DNA sequence and chromosomal fingerprint that can be used to screen for one such particularly infectious, transatlantic clone.

Pseudomonas aeruginosa accounts for up to 90% of morbidity and mortality in patients with cystic fibrosis (CF) following persistent infection over a period of years. However, during the last decade, as many as 40% of the patients in some CF centres¹⁻⁴ have also become infected with *Pseudomonas* (*Burkholderia*) *cepacia*. About 20% of the latter die from bacteremia, or aggressive pulmonary infection over a few months^{1,4}. While the significant increase in *P. cepacia* infection suggests epidemic spread⁵⁻⁸, the source and transmissibility of *P. cepacia* remains controversial⁹. Nonetheless, given the potentially grave consequence of *P. cepacia* infection, stringent infection control policies have been adopted, many CF camps in North America have been closed and all but one lung transplant centre have ceased to accept *P. cepacia*-infected CF patients as transplant candidates.

The epidemiology of *P. cepacia* infection has been examined by both ribotyping^{1,2} and pulsed-field gel electrophoresis (PFGE)-based resolution of chromosomal macro-restriction fragment length polymorphisms (RFLPs)^{3,10}. Comprehensive studies applying both methods generated two very different conclusions regarding clonality, persistence, and transmissibility. One study in the United Kingdom (Western General Hospital, Edinburgh), found a clonal relationship among isolates from 13 patients over six years¹. In contrast, during an eight-year period at a US CF centre (University of North Carolina (UNC) Hospitals, Chapel Hill), not a single identical or closely related strain was found among 23 infected clinic and lung transplant patients². Serial isolate analysis further confirmed this picture, typically demonstrating persistent infection by a single strain.

There also existed an isolate collection from another CF centre (Hospital for Sick Children, Toronto), where there was anecdotal evidence for an epidemic of *P. cepacia*. Although the isolates were not characterized for genetic relatedness, they had been uniformly resolved to express peritrichous, giant cable (Cbl)-like pili that specifically bound to CF mucin and airway epithelial cells¹¹⁻¹³. As the *cblA* pilin subunit gene encoded by all 15 of the Toronto isolates was the first such gene characterized for *P. cepacia*, a subsequent hybridization-based survey for the presence of *cblA* was carried out on multiple isolates from eight other CF centres in the United States and Europe as well as clinical and environmental strains. All of these isolates were *cblA*⁺ except for one isolate from a CF centre in Jackson, Mississippi.

Studies described in this article examine the genetic relatedness of *cblA*⁺ and *cblA*⁻ strains. The evolutionary picture generated indicates the emergence of a highly transmissible lineage, seemingly adapted for efficient transmission in the CF population. The resolved genetic markers uniquely associated with this lineage may be used to rapidly identify its presence and are therefore of immediate practical importance to CF centres in both Europe and North America.

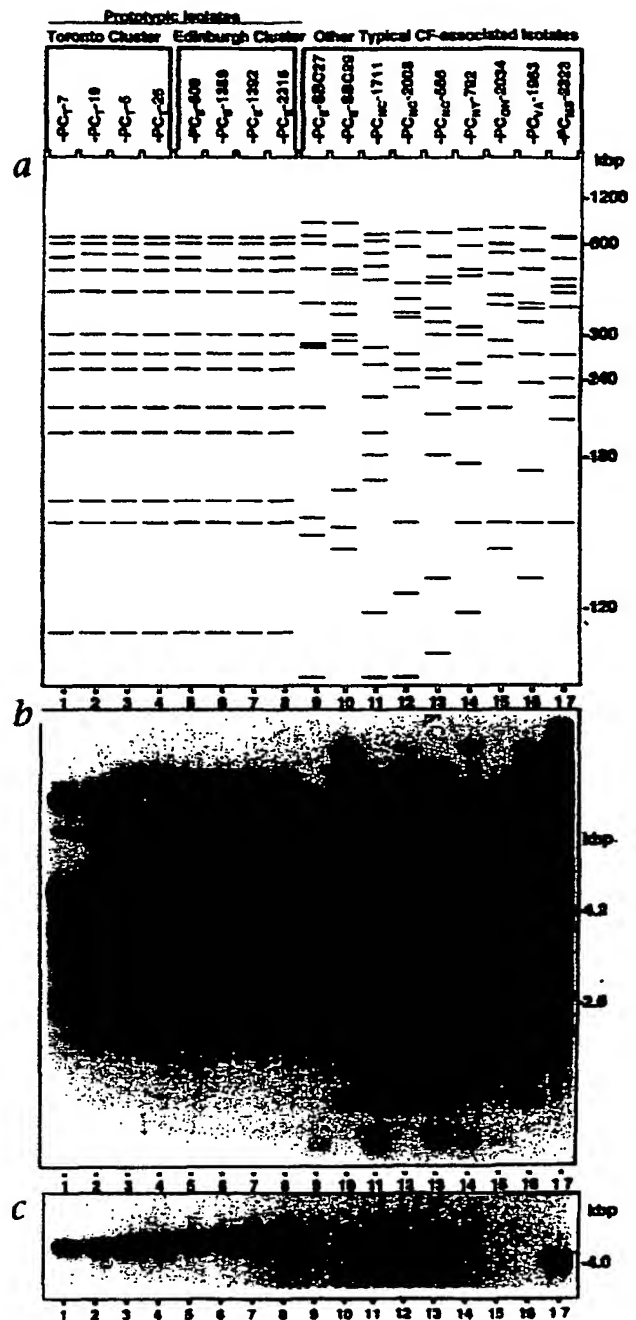
Genetic relatedness of *P. cepacia* isolates

The observations described above led us to characterize the epidemiological relatedness of the 15 Toronto isolates expressing mucin-binding Cbl pili. To investigate the genetic relationship between these isolates and those found elsewhere, we included clinical and environmental isolates as well as 78 strains from the

Fig. 1 RFLPs of 17 *P. cepacia* isolates cited in the text and Methods sections. Lane order for the 17 isolates is maintained in the three parts of the figure. Isolate numbers of examined strains appear at the top of the figure immediately above each lane. Subscript letters preceding isolate number indicate CF centre from which *P. cepacia* (PC) strain was isolated: PC_{Ed}, Edinburgh, Scotland; PC_{Jac}, Jackson, Mississippi; PC_{CH}, Chapel Hill, North Carolina; PC_{NY}, New York, New York; PC_{OH}, Cleveland, Ohio; PC_{Tor}, Toronto, Canada; and PC_{Nor}, Norfolk, Virginia. *a*, PFGE-resolved, chromosomal *SpeI* macro-RFLPs. As described previously⁹, samples were prepared and restriction fragments separated by pulsed-field gel electrophoresis with a CHEF Mapper system (Bio-Rad) through 1% agarose using a field strength of 6 V/cm and an initial and final pulse time of 1.2 s and 54 s, respectively. Fragment sizes were determined using a λ concatenate ladder (not shown). Bar-code format translation of chromosomal fingerprint profiles was made using a Macintosh Quadra 950 running Gene Construction Kit (Textco). Fragments below 100 kbp are not shown. In the latter range, Toronto and Edinburgh isolates displayed in lanes 1–8 had two identical fragments (60 kbp and 48 kbp). Other isolates (lanes 9–17) had polymorphic sets of three to six fragments in this lower range. *b*, *rm* (ribosomal RNA operon) *EcoRI* RFLPs. Southern blot hybridization methods were as we described previously²⁴ using a ³²P-labelled *rmB* probe spanning the entire *rmB* operon of *E. coli* K12. *c*, *cbIA* hybridization analysis of *EcoRI*-generated RFLPs. This was accomplished by stripping bound *rm* probe from the membrane used in Fig. 1*b* followed by hybridization with a previously described *cbIA* gene probe^{24,27}.

seven other CF centres cited above that were *cbIA*⁺. At this time the report of RFLP-identical *P. cepacia* isolates transmitted among patients at an Edinburgh CF centre appeared, and we obtained the involved strains⁹ to include in this phylogenetic characterization (Fig. 1*a*, *b*). Profiles in lanes 9–17 of both *a* and *b* of Fig. 1 depict typical polymorphic patterns resolved for isolates from different CF centres. For these isolates mean D (Dice coefficient of similarity)²⁸ for any pair by PFGE-resolved chromosomal macro-RFLP profile was 0.14 ± 0.07 (Fig. 1*a*), a level of diversity not significantly different from that found previously among eight independently isolated American Type Culture Collection (ATCC) environmental and clinical control isolates⁹. A similar degree of chromosomal RFLP variability was found between the other CF-associated isolates from the seven CF centres (results not shown), confirming that these are epidemiologically distinct strains with RFLP variability not significantly different from that of the random collection of ATCC strains ($0.1 > P > 0.05$). Despite the lower discriminatory power of ribotyping⁹, a similar degree of phylogenetic relationship among these CF-associated isolates is apparent in Fig. 1*b*.

The heterogeneity of the RFLP profiles of the isolates from the seven CF centres (for example, lanes 9–17, Fig. 1*a*, *b*) is similar to that described in a previous study involving multiple isolates from 23 patients at the UNC Hospitals CF centre⁹. This degree of variability contrasts markedly with the two closely related, conserved RFLP patterns found for the 15 *cbIA*-encoding Toronto CF centre isolates (lanes 1–4, Fig. 1*a*, *b*). Here, by examining both PFGE and ribotype RFLP profiles, the coefficient of similarity among the Toronto isolates proved to be very high, with PFGE $D = 0.95 \pm 0.03$ and ribosomal RNA operons (*rm*) $D = 0.87 \pm 0.09$. This contrasted with (*a*) the mean D value among isolates from the other seven CF centres, which was very low (for example, lanes 9–17, Fig. 1*a*, *b*), and (*b*) the mean D between the Toronto isolates and the other CF centre iso-



lates, which was also very low: PFGE $D = 0.20 \pm 0.07$, *rm* $D = 0.39 \pm 0.09$. These findings strongly suggest that all 15 of the Toronto CF centre isolates were members of a unique lineage associated with an epidemic.

Displayed in lanes 5–8 of Fig. 1*a* and *b* are *P. cepacia* PFGE and ribotype RFLP profiles of isolates from CF patients at the Edinburgh CF centre⁹. The RFLP profiles displayed in Fig. 1*a* and *b* also indicate the presence of an epidemic clone, as D for any analysed pair by either type of RFLP profile was very high (PFGE $D = 0.98 \pm 0.02$, *rm* $D = 1.0$). Further, pairwise comparison of the Edinburgh strains to the closely related Toronto CF centre strains (lanes 1–4 of Fig. 1*a*, *b*) likewise produces robust D values (PFGE $D = 0.97 \pm 0.03$, *rm* $D = 0.90 \pm 0.04$), strongly suggesting that the

Edinburgh and Toronto isolates are members of the same unique lineage despite the Atlantic Ocean barrier.

Phylogenetic relationships of *P. cepacia* isolates

Based on ribosomal RNA operon (*rrn*) RFLP profiles, phylogenetic relationships of the 133 isolates described above and in the Methods section were determined with the neighbour-joining method¹⁵. Confidence intervals on the tree topology were estimated by bootstrapping analysis¹⁶ (Fig. 2). The resultant phylogenetic tree indicates that the cluster of Toronto and Edinburgh isolates comprises a single, clonally related lineage. The remaining, independently isolated strains from other CF centres are as distantly related to one another as they are to either the Toronto/Edinburgh clusters or the independently isolated non-CF clinical and environmental strains.

Cbl phenotype and genotype of Edinburgh CF isolates

We then examined the epidemic Edinburgh isolates to see if, as do the Toronto strains⁴, they expressed Cbl pili and encoded the *cblA* gene. Phenotypic survey using electron microscopy revealed that these highly transmissible strains expressed appendage pili that were structurally equivalent to those expressed by all of the *cblA*⁺ Toronto isolates^{4,13} (Fig. 3). Genotypic survey was carried out by stripping *rrn*-probe from an *Eco*RI chromosomal digest membrane (Fig. 1b) followed by hybridization with a *cblA* probe. The highly transmissible Edinburgh isolates as well as the closely related Toronto clones encode *cblA* (Fig. 1c, lanes 1–8).

cblA gene sequence-based test of clonality

Because the implications of our studies have the potential to in-

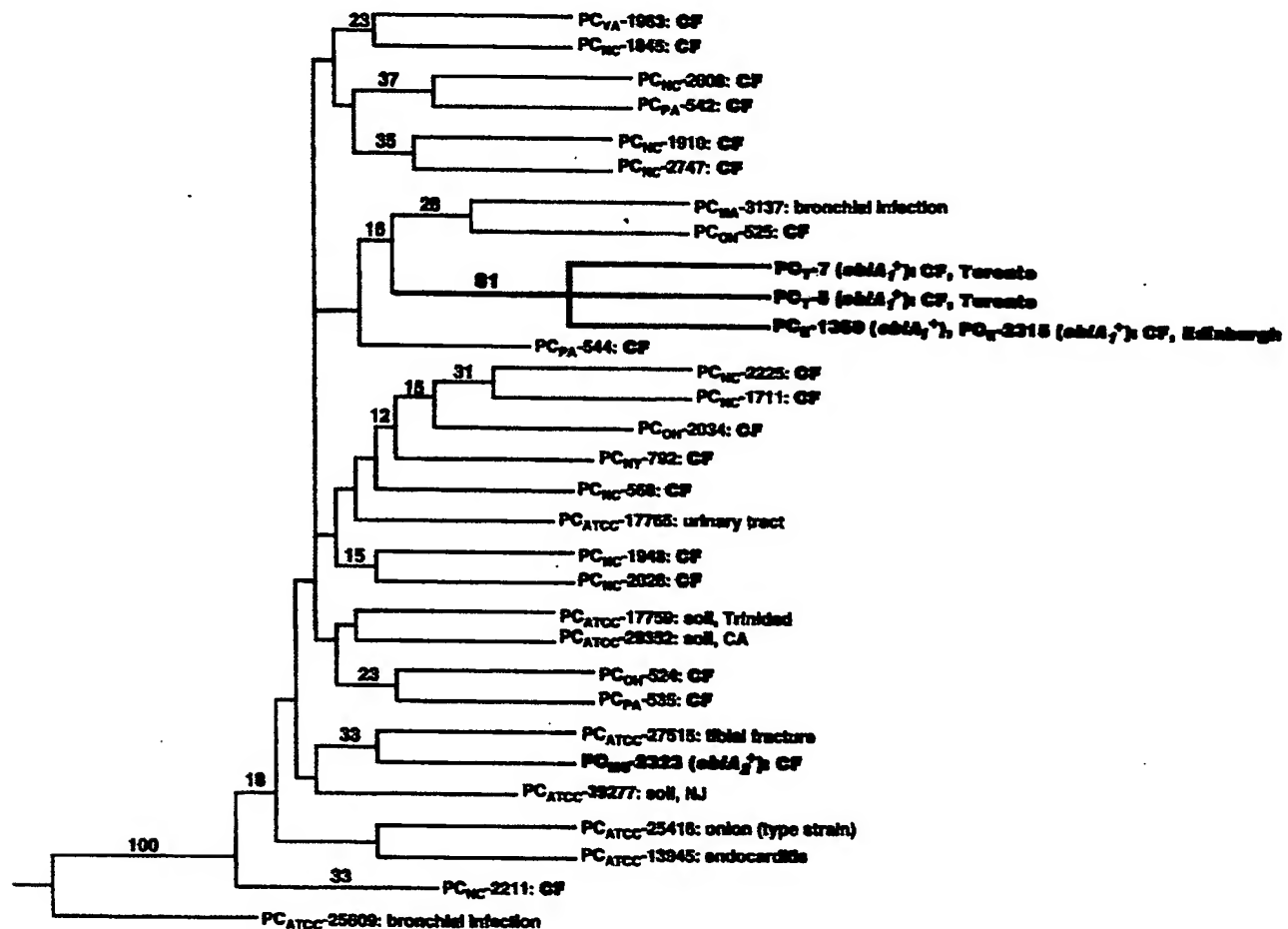


Fig. 2 *rrn*-RFLP-based phylogenetic tree of representative isolates from patients at seven CF centres in North America (Chapel Hill, North Carolina; Jackson, Mississippi; Norfolk, Virginia; Cleveland, Ohio; Philadelphia, Pennsylvania; New York, New York; Toronto, Ontario) and the United Kingdom (Edinburgh) plus environmental and clinical (non-CF) sources. All cited isolates are described in the text and in the Methods section. Indicated isolate number is followed by source (CF, environmental or clinical). *cblA*⁺, *cblA*⁻, isolate(s) that encode the *cblA* gene (Fig. 1c) and express adhesin Cbl pili (Fig. 3). *cblA*⁺, identical 501-bp sequence carried by Toronto and Edinburgh CF centre isolates (Fig. 4); *cblA*⁻, polymorphic 501-bp sequence carried by Jackson, Mississippi, CF centre isolate PC_{NC}-2323 (Fig. 4). Number above each branch indicates the percentage of time each was joined together under bootstrap analysis¹⁶ (confidence intervals less than ten have been omitted for clarity). The lineages included in this tree are representative of the larger sample of isolates collected. Multiple CF patient serial isolates of an identical *rrn* RFLP profile have not been included as they do not affect the tree topology. However, multiple isolates from Toronto (PC₋5, PC₋7) and Edinburgh (PC₋1359, PC₋2315) CF centres are noted because further analysis by DNA sequence revealed that the *cblA* genes encoded by these four isolates are identical (Fig. 4). The remaining 13 and 11 isolates, respectively, from each of these two CF centres are members of the indicated epidemic lineage based on 100% correlation of their *rrn* RFLP profiles with those of the prototypic patterns of the Toronto/Edinburgh isolates shown in Fig. 1b.

fluence directly clinical management of some 70,000 CF patients in Europe and North America, we used DNA sequence analysis to test the RFLP-based conclusions that isolates from Toronto and Edinburgh were clonal. Either of two classes of bacterial genes are typically sequenced for this purpose: slowly evolving, 'house-keeping' genes such as *putP* (proline permease)¹⁷ or, more rapidly evolving, antigen-encoding genes such as the flagellar filament gene (*flaC*) of *Salmonella typhimurium*¹⁸. Based on analogy to the latter, we chose the *cblA* pilin gene as it would probably be under antigenic selection, thus providing a more rigorous test for the clonality of strains. The possibility of antigenic variability being reflected in the *cblA* pilin gene sequence was suggested by our analyses of the Jackson, Mississippi, CF-associated strain PC_{ms}-2323, the sole *cblA*-positive isolate not associated with the epidemic clusters (lane 17, Fig. 1c). Variability in the encoded *cblA* of this isolate had been inferred by (1) ribotype and chromosomal macro-RFLP profiles indicating that PC_{ms}-2323 was only distantly related to the *cblA*-positive Toronto/Edinburgh isolates (lane 17 versus lanes 1–8 of Fig. 1a, b); (2) variation in *cblA*-encoded restriction fragment size from that observed in the Toronto/Edinburgh isolates (lane 17 versus lanes 1–8 of Fig. 1c) and (3) absence of agglutination by antibodies made against Cbl pilin purified from *cblA*-positive Toronto CF centre isolates (data not shown).

Primers were synthesized from the *cblA* sequence of the Toronto isolate PC_r-7 (ref. 4) and used for polymerase chain reaction (PCR)-based amplification of the *cblA* gene from isolates to be characterized. Resultant PCR products were then cloned and sequenced (see Methods). Complete *cblA* sequences were thus obtained from isolates with the two slightly variant though closely related RFLP profiles typical of the 15 Toronto CF centre isolates (Fig. 1a, b, lanes 1–4), the two slightly variant though closely related RFLP profiles typical of the 13 Edinburgh CF centre isolates (Fig. 1a, b, lanes 5–8), and the significantly variant Jackson, Mississippi, CF-associated strain PC_{ms}-2323 (Fig. 1a, b, lane 17). Comparison of these five sequences indicates that the chromosomally encoded, 501-base pair (bp) *cblA* pilin subunit structural gene carried by the closely related isolates from the Toronto and Edinburgh CF centres was invariant in sequence. In contrast, the *cblA* gene encoded by the distantly related Jackson Mississippi strain PC_{ms}-2323 exhibited polymorphism at the sequence level, with changes in 60 bp of the 501-bp sequence (88% identity; see Fig. 4). **The perfect conservation of the *cblA* pilin sequence among isolates from multiple patients over four years at the Toronto and Edinburgh centres is precisely what would be expected for epidemic transmission of a highly infectious clone. Likewise, the variant *cblA* encoded by the Mississippi CF centre isolate is in accord with that expected for a distantly related isolate (Fig. 2).**

Discussion

P. cepacia varies in transmissibility

Consideration of these findings in the context of our previous studies on UNC CF centre isolates⁴ leads us to conclude that isolates of *P. cepacia* are not equally transmissible between CF patients, rather, there exists at least one significantly divergent, highly transmissible clonal lineage plus numerous moderately heterogeneous lineages of negligible transmissibility (see Fig. 2), and that the highly transmissible lineage identified is responsible for epidemics at North American and British CF centres. This was most likely due to an as yet unidentified transatlantic transmission related to joint summer camp attendance.

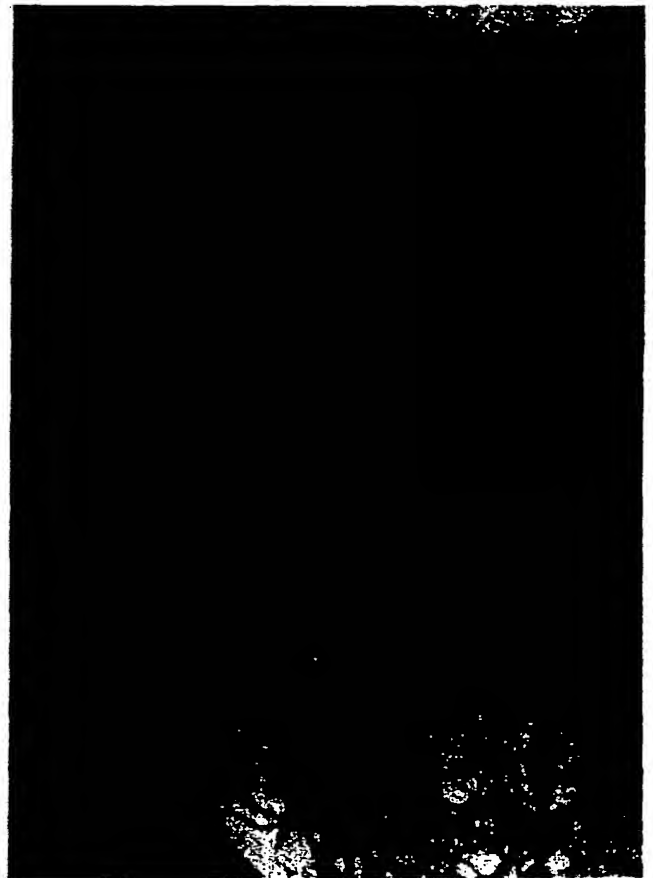


Fig. 3 Transmission electron micrograph of Toronto epidemic strain PC_r-7 expressing Cbl adhesin pili. High resolution was achieved with a JOEL 100 CX electron microscope as previously described¹³. Bar in lower right corner, 0.1 μ m. Taken from Goldstein et al¹³.

Relation of Toronto/Edinburgh clone to other epidemic strains

Based on *rrn* RFLP profiles or anecdotal evidence, additional reports suggest the occurrence of *P. cepacia* transmission at CF centres in Philadelphia⁹ and Cleveland¹⁰, respectively. We characterized strains involved with both of these putative outbreaks (see Methods section) and found that by neither ribotype or macro-chromosomal RFLPs profile did the prototypic RFLP fingerprints of the putatively epidemic strains from either centre appear similar to one another (mean $D \leq 0.3$), nor to the unique, highly transmissible lineage involved with the Toronto and Edinburgh CF patients (L.S., A.H., H. Zhou and R.G., unpublished data). Nonetheless, highly conserved RFLP profiles (mean $D \geq 0.85$) within the individual outbreaks did support a picture of epidemic transmission within each of the two centres. When these isolates were further characterized, hybridization-based survey for the presence of the *cblA* pilin gene proved negative for 35 of the involved strains (L. S., A.H., H. Zhou and R.G., unpublished data). These results suggest that there may exist *P. cepacia* lineages of high transmissibility other than the *cblA*⁺ clone that we have identified.

Emergence of clone for efficient infection of the CF lung

This study demonstrates the integral role of molecular epidemi-

ology and evolutionary biology in identifying newly emerging, highly transmissible microbial pathogens. The degree of divergence of the *cblA*⁺ lineage suggests specific adaptive, evolutionary changes for efficient transmission in the CF population. A number of phenotypic observations support this hypothesis, such as the novel giant Cbl pili expressed, which have been found to promote adhesion-based colonization of the CF airway^{41,42}. New results provide further details, showing that *cblA*⁺ isolates are significantly more adherent to human, primary culture *cfr*⁻ airway epithelial cells than *cblA*⁻ strains, and that the *cblA*⁺ isolates adhered to the epithelial apical surface as well as to the cilia (J. Yankaskas, P. Gilligan and R.G., unpublished observations), suggesting the potential for interference with the mucociliary transport system. Further, unlike non-epidemic *P. cepacia* isolates, clones from the Toronto/Edinburgh epidemics proved uniquely resistant to killing by *P. aeruginosa* isolates cultured from many different patients (C. Campanelli, A.H. and R.G., unpublished observations). Given that CF patients are most often infected with *P. aeruginosa* before superinfection by *P. cepacia*, this atypical resistance may contribute to the remarkable capacity of the *cblA*⁺ lineage to be epidemically spread among the CF population.

PC₁₀₅-2323. From each of these *cbiA* probe-positive chromosomes, the *cbiA* gene was PCR amplified²¹ using a DNA thermocycler (Perkin-Elmer) with a GeneAmp PCR Core Reagents Kit (*ibid.*). Based on the previously determined sequence of the *cbiA* gene encoded by isolate PC₁₀₅-7 (ref. 4), sense and antisense primers used for these reactions were 5'-CCAAAGGACTAACCCA-3' and 5'-ACGCGATGTCCATCACA-3', respectively. PCR reactions were as follows: cycle one, 2 min at 94 °C, 2 min at 37 °C, 1 min at 72 °C. The remaining 29 cycles were: 1 min at 94 °C, 1 min at 45 °C, 1 min at 72 °C, followed by 7-min extension at 72 °C. PCR products were separated by electrophoresis through 0.8% agarose and for each a single band was observed with ethidium bromide staining. Bands were electroeluted into DEAE membrane (Schleicher & Schuell) and cloned with a TA Cloning Kit (Invitrogen). DNA sequences were determined by the Sanger dideoxy method²² with the same primers used for PCR amplification (above). Five PCR-amplified *cbiA* gene clones of PC₁₀₅-2323 were generated, three of which were sequenced for confirmatory purpose, with no variation resolved.

Statistics. Standard criteria were used for comparing PFGE patterns²³. According to established criteria for *P. cepacia*²⁴, strains were assigned to the same ribotype when comparison of sizes of hybridizing fragments revealed three or fewer bands differing between the two patterns under comparison. Quantitative pairwise comparison of both types of RFLP patterns was accomplished using the Dice coefficient of similarity calculated as $D = 2n_{xy} / (n_x + n_y)$, where n_x is the total number of DNA fragments from strain X, n_y is the total number from strain Y, and n_{xy} the number of fragments identical in the two strains^{14,25}. The coefficient of similarity for two PFGE RFLPs $D \geq 0.90$ represents closely related strains, while unrelated strains have $D \leq 0.60$. Intervening values, remarkably, are rare². For *rrn* RFLPs, given that *P. cepacia* strains typically display 7–10 distinct hybridizing bands, the shared ribotype (above) would correspond to $D = 0.79$ to 0.85. Comparisons between mean values were performed by Student's *t*-test using a Systat program (Systat Inc.).

Acknowledgements

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Identification of IS1356, a New Insertion Sequence, and Its Association with IS402 in Epidemic Strains of *Burkholderia cepacia* Infecting Cystic Fibrosis Patients

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Burkholderia cepacia is now recognized as an important opportunistic pathogen in cystic fibrosis (CF) and other compromised patients. Epidemicity among CF patients has been attributed to at least one particularly infectious strain (strain ET12), and both genetic evidence and anecdotal evidence suggest that this strain, currently endemic in Ontario, and those causing an epidemic in the United Kingdom, are indeed the same. Our study was conducted to determine whether there was any association between the presence of various insertion sequence (IS) elements, the cable pilin subunit gene (*cblA*), electrophoretic type (ET), and ribotype (RT) in a collection of 97 clinical and 2 environmental isolates of *B. cepacia*. No apparent linkage was found for IS elements IS401, IS402, IS406, IS407, and IS408 with ET or RT. The *cblA* target, said to be a marker for high infectivity, was detected in 100% (38 of 38) of strains of *B. cepacia* ET12 and in a single strain of ET13 that differed in a single enzyme allele. A new IS, IS1356, identified during the investigation, was present in 71.7% of all isolates, and 50.7% of these isolates harbored IS1356 as a hybrid IS element inserted into IS402. IS1356 is 1,353 bp in length, and when it is inserted into IS402 it results in a 10-bp duplication at the site of insertion. IS1356 contains one major open reading frame of 1,260 bp coding for a putative transposase which has significant homology to IS*Rm3* in *Rhizobium meliloti* (59%) and to an undesignated IS element in *Corynebacterium diphtheriae* (49%). The IS402-IS1356 element was found exclusively in the epidemic strains from Ontario and the United Kingdom, being detected in 94.7% (36 of 38 isolates) of *B. cepacia* ET12 isolates. Of the two ET12 isolates found to be devoid of the IS402-IS1356 element, both contained IS1356 unassociated with IS402, one was temporally unrelated to the epidemic, and the other was from a CF patient in a geographic area remote from Ontario and the United Kingdom. It is evident that the IS402-IS1356 hybrid element, the *cblA* pilin subunit gene, and the allelic suite represented by multilocus enzyme electrophoretic type ET12 may provide useful markers for the epidemic, highly transmissible transatlantic strain isolated in Ontario and the United Kingdom.

Burkholderia cepacia is an aerobic gram-negative bacillus commonly found throughout the environment and as a phytopathogen causing soft rot in onions (1). Over the past decade, however, strains which cause opportunistic infections in humans, most notably in cystic fibrosis (CF) patients, have been encountered with increasing frequency, leading to an increase in morbidity and mortality (12, 38). Among non-CF patients extrapulmonary nosocomial infections have more recently been reported (21).

Although the mechanism of virulence of *B. cepacia* isolates has not been elucidated (19), isolates from CF patients have been shown to adhere to mucin (26) and buccal epithelial cells (27). There may also be a correlation between the source of *B. cepacia* isolates (e.g., environmental and CF-associated epidemic and nonepidemic isolates) and the particular class of pili expressed (9). The implications of being colonized with *B. cepacia* isolates are a growing concern in the CF patient community, and markers of strain virulence are eagerly sought. Enhanced transmissibility and virulence appear to be strain dependent, and epidemic lineages are being defined anecdot-

ally and genetically (10, 13, 17, 33–36). To date, studies have indicated cross-infection between patients (10, 17, 25, 31) and nosocomial acquisition (20) as important parameters of transmission.

In attempts to limit the spread of *B. cepacia* strains, many clinical centers now segregate colonized and noncolonized CF patients. This has proved to be successful but is limited by the social contacts between patients outside of the hospital setting that is the norm for CF patient groups, especially adults (10, 17, 31).

Many studies involving *B. cepacia* strains have focused on their truly extraordinary potential to metabolize a wide variety of organic compounds. It is currently thought that this metabolic versatility may, in part, be the result of the genomic complexity (24) and the large number of insertion sequence (IS) elements identified in *B. cepacia* strains (7, 15). IS elements have the ability to promote genomic rearrangement, recruit foreign genes, and cause insertional gene activation. Indeed, most of the IS elements in *B. cepacia* isolates have been identified by observing these features (16). The effect of IS elements on the genes with which they are associated is well documented (8), and it is conceivable that they may act genetically to increase the transmissibility and pathogenicity of certain strains of *B. cepacia*.

We originally identified the strains obtained by Govan et al. (10) in 1993 and ourselves (13, 25), from the United Kingdom

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TABLE 1. Oligonucleotide primers used to detect the various IS elements and *cblA* pilin subunit gene in *B. cepacia*

Target gene	Primer sequences	Amplicon size (bp)
IS402	A: 5'-CAA CCG AGA CTG AGG AGA TG-3' B: 5'-GCT GCT TGC CAA TCG CGC TC-3'	250
IS406	A: 5'-GAC GGT GGG TCT GAC GCC AT-3' B: 5'-AAG CCC TGA GTC CCT CGT CG-3'	450
IS407	A: 5'-TCA TCG GGT TTC TGA AGG AA-3' B: 5'-CGG AAG CGA GCT GCA CGG TC-3'	750
IS408	A: 5'-TTG AAG GAA GTC CTG CGA CT-3' B: 5'-TCG ACT TCG CCC AAT CCT TG-3'	370
IS1356	A: 5'-GGC CCT GAA GAA GGC GAT AT-3' B: 5'-TCC GGC GAC ACC TCG ATG CC-3'	327
<i>cblA</i> ^a	A: 5'-CCA AAG GAC TAA CCC A-3' B: 5'-ACG CGA TGT CCA CA-3'	610

^a Primers were first described by Sajjan et al. (28).

and Canada, respectively, as having an identical enzyme electrophoretic allotype (electrophoretic type 12 [ET12]), the first direct evidence that the anecdotal association of Canadian *B. cepacia* strains currently endemic in Ontario and those causing an epidemic in the United Kingdom were the same. In the present study, the frequencies of occurrence of various IS elements were studied in our collection of clinical *B. cepacia* strains to determine if any relationship exists between these genetic modifiers and electrophoretic type (ET) or ribotype (RT). Additionally, a recent publication by Sun et al. (36) described the presence of novel cable pili in the epidemic clone described above, and our collection of strains was therefore also screened for the presence of the *cblA* pilin subunit gene.

MATERIALS AND METHODS

Bacterial strains, nucleic acid preparation, ribotyping, and multilocus enzyme electrophoresis. The collection of strains used in the investigation consisted of 99 isolates of *B. cepacia*, most of which were previously characterized for their ETs and RTs (13). Strains were grown overnight on Columbia blood agar base (Quelabs, Montreal, Quebec, Canada) at 37°C in 5% CO₂ prior to nucleic acid (NA) extraction. The isolates were originally obtained as isolates either from CF patients or from nosocomial outbreaks, and they were maintained in the culture collection of the Laboratory Centre for Disease Control, Ottawa, Ontario, Canada. Two of the strains investigated were American Type Culture Collection (ATCC) reference strains of environmental origin (ATCC 17759 and ATCC 25416). Procedures for NA purification, ribotyping, and multilocus enzyme electrophoresis were as described previously (13).

Oligonucleotide primers and PCR amplification. The sequences of the oligonucleotide primers designed to detect the various IS elements and the pilin subunit gene are summarized in Table 1 and are based on the published sequences for IS402 (6), IS406 and IS407 (41), IS408 (2), and *cblA* (36). Primers for the detection of IS1356 were designed on the basis of the sequence data acquired in the present investigation. All primers were synthesized on a 392 DNA-RNA Synthesizer (Applied Biosystems, Foster City, Calif.) by using standard phosphoramidite chemistry. Amplification was performed in a PE9600 thermocycler (Perkin-Elmer Cetus, Foster City, Calif.), with PCR mixtures containing 0.2 mg of NA per ml, 200 mM deoxynucleoside triphosphate, 1 mM (each) primer in the pair, 50 U of *Taq* polymerase (Boehringer Mannheim, Laval, Quebec, Canada) per ml, and 1× reaction buffer supplied by the manufacturer. Thermocycling

conditions consisted of an initial denaturation of 2 min at 94°C; this was followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. Following amplification, the samples were incubated at 72°C for 10 min and were then cooled to 4°C. Amplicons were detected by electrophoresis in 2% agarose and staining with ethidium bromide (29).

Vectorette PCR was performed as described previously (23) by using primer IS1356-A (Table 1) as the target primer. Vectors libraries were constructed with NAs from strain LCDC 92-498 (ET12, RT20) for *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Hind*III, *Nhe*I, *Sal*I, *Spe*I, and *Xba*I. This isolate is a member of the group of strains implicated in the spread of *B. cepacia* ET12 between the United Kingdom and Canada (10, 13, 31). Amplification was performed as described above by using a two-step thermocycling profile of 30 cycles of 94°C for 30 s and 72°C for 3 min. The reaction mixtures were analyzed on a 1% low-melting-point agarose gel, and the resulting amplicons were excised from the gel and purified with the Wizard PCR Prep Purification system (Promega, Madison, Wis.).

Cloning and sequencing of IS402-IS1356. A bacteriophage library was constructed from strain LCDC 92-498 by using the ZAP Express Cloning Kit (Stratagene, La Jolla, Calif.). The probe was prepared by amplifying NA from strain LCDC 92-498 with the primers IS1356-A and IS1356-B (Table 1) in the presence of digoxigenin-11-uridine-5'-triphosphate (DIG). PCR conditions were identical to those used to detect the IS; however, the deoxynucleoside triphosphates were substituted with DIG Labeling Mix (Boehringer Mannheim). Positive clones were identified with the DIG DNA Detection Kit (Boehringer Mannheim) according to the manufacturer's directions. After purification of the bacteriophage clones, the phagemids were excised as directed in the ZAP Express Kit and plasmid DNA was purified with the QiaWell Plus Plasmid purification system (Quiagen, Chatsworth, Calif.) as recommended by the manufacturer.

Sequencing was performed on an ABI 373 automated DNA sequencer by using the Prism Dye Terminator sequencing kit (Applied Biosystems). Sequencing primers were designed on the basis of the acquired data as required to complete the sequence. Sequence analysis was performed with the various programs supplied with PG/Gene (Intelligenetics, Mountain View, Calif.) and LaserGene (DNASar, Madison, Wis.). Phylogenetic analysis was performed with PAUP, version 3.0 (37).

IS designation and nucleotide sequence accession number. The IS1356 designation was from Esther M. Lederberg (Stanford University School of Medicine, Stanford, Calif.), under the auspices of the Plasmid Reference Centre Prefix Registry.

The IS402-IS1356 sequence has been assigned GenBank accession number U44828.

RESULTS

Frequency of targeted IS elements in *B. cepacia*. The frequencies of occurrence of targeted IS elements in *B. cepacia* isolates from environmental, nosocomial, and CF patient sources are recorded in Table 2. Overall, IS402 was found in 68.7% of the isolates, IS406 was found in 22.2% of the isolates, IS407 was found in 48.5% of the isolates, IS408 was found in 53.5% of the isolates, and IS1356 was found in 71.7% of the isolates. In addition to these IS elements, primer sets were also designed to amplify IS401 (2), but no amplicons were detected in this collection of isolates (data not shown). There were no apparent linkages between the presence of these IS elements and an ET or an RT, with one notable exception. The primers targeting IS402, in addition to detecting the IS element, also primed an additional amplicon of approximately 650 bp in some isolates. This anomaly was restricted to strains designated ET12, which is the ET of isolates documented to be highly transmissible in CF patients (10, 13, 31). Sequence data revealed that the 650-bp amplicon consisted initially of the

TABLE 2. Distribution of IS elements in *B. cepacia* isolates

Source	No. of isolates	No. (%) of isolates with the indicated IS element					
		IS402	IS406	IS407	IS408	IS1356	IS402-IS1356
Environmental	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	0 (0.0)
Nosocomial	15	10 (66.7)	7 (46.7)	2 (13.3)	12 (80.0)	4 (26.7)	0 (0.0)
CF patients	82	58 (70.7)	15 (18.3)	46 (56.1)	41 (50.0)	66 (80.5)	36 (43.9)
Total	99	68 (68.7)	22 (22.2)	48 (48.5)	53 (53.5)	71 (71.7)	36 (36.4)

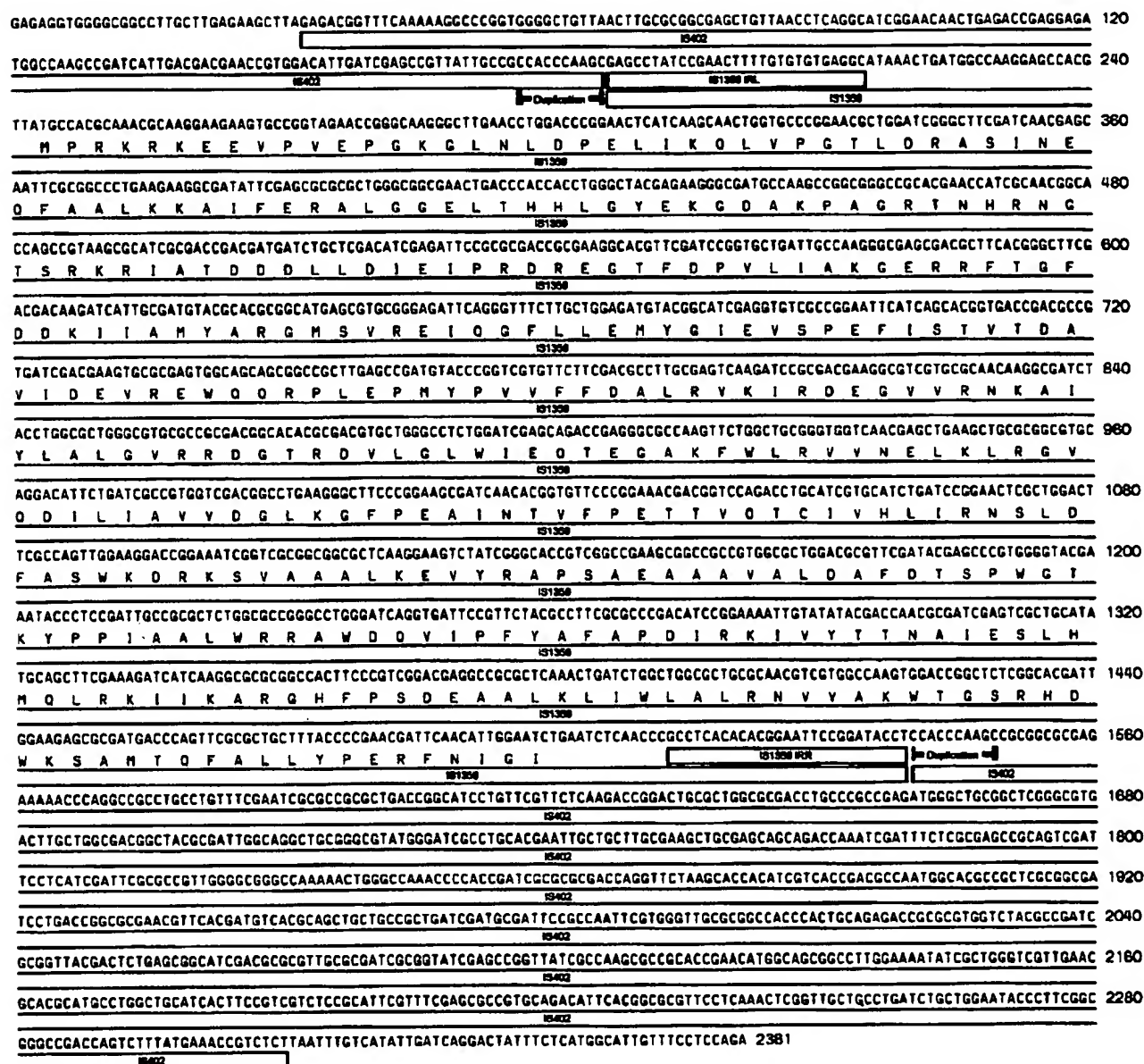


FIG. 1. Nucleotide sequence of IS402-IS1356 and the amino acid sequence of the putative transposase of IS1356. The pertinent features of the IS402-IS1356 element are illustrated below the nucleotide sequence. The locations of IS402 and IS1356 and their terminal inverted repeats are shown by the open boxes. The 10-bp duplication of IS402 due to the insertion of IS1356 is indicated by the solid bars.

IS402 sequence, but this was interrupted after 154 bp and was succeeded by the sequence of IS1356.

Cloning and characterization of IS402-IS1356. Through the use of the vectorette PCR, an amplicon of approximately 1,300 bp was obtained from the *Bcl*I library and was used for sequencing. The *Bgl*II and *Eco*RI libraries also produced amplicons, but these were considerably smaller in size and were not investigated further. After sequencing of the amplicon, further attempts at "gene walking" through the use of vectorette PCR proved unsuccessful because of the large number of unresolvable amplicons obtained. From the bacteriophage library 10 candidate clones were selected for sequencing and allowed for the identification of three different insertion sites. Insertion into IS402 is shown in Fig. 1. The two other sites identified

were 5'-CTGACCGGCGG-IS1356-CCACCGGTGA-3' and 5'-CGTTGTCTCG-IS1356-3'. The clone containing the latter insertion site did not contain the full IS1356 sequence, and therefore, the 3' insertion sequence is not known.

The IS402-IS1356 element consists of the IS402 reported by Ferrante and Lessie (6), including the 3-base duplication (5'-TTA-3') at the insertion site. Although certain sequence differences were detected between the IS402 sequence reported previously and the one present in the hybrid, these differences were not considered significant. The IS402 sequence is interrupted after 154 bases by IS1356, resulting in a 10-bp duplication at the insertion site. IS1356 is 1,353 bp in length and terminates at either end by imperfect inverted repeats. The left-hand inverted repeat (IRL) is 27 bp in length and the

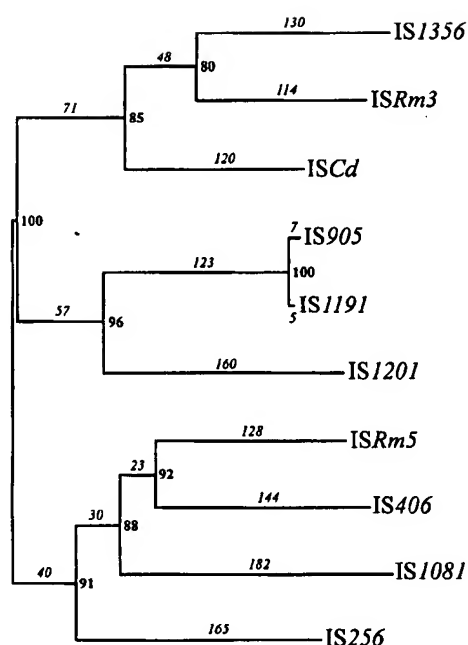


FIG. 2. Phylogenetic relationships among the putative transposases of various IS elements and that of IS1356. The dendrogram was generated after 100 bootstrap replications of branch-and-bound searches by using PAUP, version 3.0. The numbers along the branches indicate the branch lengths, and the numbers at the branch nodes are the respective bootstrap values. The transposases compared with that of IS1356 were those found on ISRm3 from *R. meliloti* (40), ISCd from *Corynebacterium diphtheriae* (22) (ISCd is an unofficial designation for this element), IS905 from *Lactococcus lactis* (5), IS1191 from *Streptococcus thermophilus* (11), IS1201 from *Lactobacillus helveticus* (39), ISRm5 from *R. meliloti* (14), IS406 from *B. cepacia* (41), IS1081 from *Mycobacterium bovis* (4), and IS256 from *Staphylococcus aureus* (3).

right-hand inverted repeat (IRR) is 29 bp in length, with seven mismatches over the common region. IS1356 contains one major open reading frame of 1,260 bp which codes for a putative transposase. This transposase showed significant homologies to several others found in the Swiss-Prot 31 database, with the most significant homologies of 59% to ISRm3 found in *Rhizobium meliloti* (40) and 49% to an undesignated IS found in *Corynebacterium diphtheriae* (22). The phylogenetic relationships between several of the more closely related transposases are shown in Fig. 2.

In order to determine if the IS402-IS1356 element had a conserved insertion site, primers which would amplify either the 5' or 3' insertion sites were designed, and the resulting amplicons were sequenced (data not shown). All isolates in which the IS402-IS1356 hybrid IS element was detected yielded an amplicon of the predicted size with a sequence identical to that originally identified.

Distribution of targeted IS elements and *cbiA* pilin subunit genes among several *B. cepacia* ETs. Table 3 summarizes the distributions of the IS elements and the *cbiA* pilin subunit genes in our collection of 99 strains representative of 20 ETs. When IS1356 was detected in strains, the primers did not permit discrimination between IS elements found as the hybrid and IS1356 located at other sites. The initial identification of the IS402-IS1356 element was accomplished by observing the 650-bp band obtained with the IS402 primer set; however, in these strains successful amplification of the target was difficult to reproduce, so the presence of the hybrid was confirmed by screening all isolates with the IS402-A and IS1356-B primers to obtain a 592-bp amplicon (data not shown).

Two of the ET12 isolates studied were found to lack the IS402-IS1356 element. One of these isolates was found to possess all of the other IS elements investigated except IS406, and the other possessed IS408 and IS1356. Neither of these two ET12 isolates lacking the hybrid element were clearly associated with the epidemic, in that one is a reference strain used many years ago to establish the serotyping scheme for *B. cepacia* (18) and the other came from a CF patient resident in

TABLE 3. Distribution of targeted genes in *B. cepacia* strains representing 20 ETs

ET	No. of isolates tested	Source ^a	No. of isolates with the indicated IS or gene						
			IS402	IS406	IS407	IS408	IS1356	IS402-IS1356	<i>cbiA</i>
1	1	CF	1	0	1	1	1	0	0
2	1	CF	1	0	1	0	0	0	0
3	1	NS	1	0	0	1	0	0	0
4	2	CF	0	0	0	0	1	0	0
5	1	CF	0	0	1	0	0	0	0
6	3	CF	0	0	0	0	2	0	0
7	1	CF	0	0	0	0	0	0	0
8	2	NS	2	1	2	1	0	0	0
9	1	ENV	0	0	0	0	0	0	0
10	1	NS	0	0	0	1	0	0	0
11	5	NS	1	3	0	4	2	0	0
12 ^b	38	CF	37	10	37	32	38	36	38
13 ^c	1	CF	1	0	1	0	1	0	1
14	1	CF	0	0	1	0	1	0	0
15	1	NS	1	0	0	1	1	0	0
16	18	CF	9	2	2	1	18	0	0
17	12	CF	10	2	2	7	5	0	0
18	4	NS	4	4	0	4	0	0	0
19	1	ENV	0	0	0	0	1	0	0
20	4	CF	0	0	0	0	0	0	0

^a CF, CF patient; ENV, environmental; NS, nosocomial.

^b Epidemic transatlantic clone (Canada and the United Kingdom).

^c Isolate from a Canadian CF patient in a province remote from Ontario and with no known epidemic association.

a remote area of northern Ontario with no known association with areas of the provincial epidemic in the south. The *cblA* pilin subunit gene was found in all ET12 isolates tested and one strain of ET13 from an adult CF patient in a province remote from Ontario. There is no documented evidence of an epidemic association or the spread of this single ET13 isolate, which differs in only one esterase allele from ET12 and which is IS402-IS1356 negative (Table 3). The *cblA* amplicon from this isolate was subjected to sequence analysis and was found to be identical to that in the ET12 isolates (data not shown).

DISCUSSION

In our earlier study (13), we demonstrated that while RTs appear to be highly variable in a geographical context, ETs seem relatively stable in a population of *B. cepacia* isolates from particular clinical sources and may, in fact, be the best indicator of a clonal distribution. Considering the large number of ISs known to reside in *B. cepacia* strains and their propensity to cause genetic rearrangements, the variability of RTs is not surprising. Since a number of IS elements found in *B. cepacia* isolates have been shown to affect the expression of associated genes in other circumstances (6, 16, 30, 41), we questioned if there were any linkages between carriage of particular IS elements, transmissibility, and/or virulence and ET type.

During the course of our investigations we succeeded in identifying a previously unreported IS element, which has been designated IS1356. This sequence has a structure typical of ISs in that it is terminated by inverted repeats, contains an open reading frame which spans virtually its entire length, and codes for a putative transposase. An interesting feature of IS1356 is the high degree of similarity of the transposase to a variety of others identified on IS elements from a wide distribution of organisms. During the characterization of IS*Rm5*, Laberge et al. (14) observed that there appears to be a family of IS elements, of which IS1356 is now a member, which share a common ancestry, even though the hosts of these IS elements are from highly divergent bacterial families. From an evolutionary standpoint these similarities are quite intriguing. Similarities between IS elements from *B. cepacia* and *R. meliloti* strains could possibly be explained by their close association with respect to environmental habitat, but the ancestral relationship between these organisms and the other members of this family is difficult to fathom. Another interesting feature of IS1356 is its insertion into IS402. While this arrangement is not unprecedented (14, 32), it proved to be unique to a particular clonal cluster of *B. cepacia* (the ET12 cluster) and was not found in other isolates. In addition, the site of insertion of the IS402-IS1356 element was identical in all of the isolates investigated, further supporting the clonal nature of this group of isolates.

Although a large number of IS elements have been identified in *B. cepacia* strains, our study of their distribution was limited by the fact that only a small number of these have been sequenced. For those for which sequence data were available, no relationship between ET, RT, and IS carriage was found with the exception of the association of the IS402-IS1356 element with ET12. The IS402-IS1356 element was only found in *B. cepacia* strains isolated from CF patients and was restricted to ET12, with 36 of 38 (94.7%) of these isolates harboring the hybrid. Two of the 38 ET12 isolates examined lacked the IS402-IS1356 element, although they both contained IS1356 alone. Neither strain could be directly linked to the epidemic clone, because one was an early isolate used to establish a serotyping scheme and therefore was temporally unrelated and the other was isolated from a CF patient living in a remote

location in Ontario with no obvious connections to the urban epidemic in that province.

The current study did not directly address this issue; however, it was noted that there appeared to be a certain bias in the distribution of IS elements among the clinical isolates with respect to the source of the host from which the strains were isolated. The IS elements IS406 and IS408 appeared more frequently in nosocomial isolates, whereas the IS elements IS407 and IS1356 appeared more frequently in isolates from CF patients. IS402 was equally distributed between the two groups, and the IS402-IS1356 element was found exclusively in CF patients and was restricted to ET12 isolates. Admittedly, the number of nosocomial isolates investigated was low and a wider sampling may alter the apparent bias in IS element distribution; however, it is also possible that this bias may reflect an ancestral branching of *B. cepacia* strains with respect to their clinical significance and association with a particular disease manifestation.

At present it is not known if the IS402-IS1356 element is linked to the apparent increase in transmissibility and virulence of *B. cepacia* ET12, only that there is an "association." It is quite possible that the hybrid IS is simply a marker for this particular lineage of *B. cepacia*. Although the present study was not designed to demonstrate a direct effect of IS elements on virulence, the possibility of this type of association is not precluded. The importance of IS elements in the adaptability of *B. cepacia* isolates should not be ignored, and while they may not be directly related to virulence, they may, nonetheless, play an important indirect role in pathogenicity by assisting in the acquisition of virulence factors in certain strains.

The pathogenicity of *B. cepacia* and the implications of colonization with this organism are of considerable interest to those involved in the management of CF patients. Anecdotal evidence has recently linked a highly transmissible ET12 clone currently endemic in Ontario, Canada, to that causing an epidemic in the United Kingdom (10, 13). Sun et al. (36) demonstrated that the infectious, transatlantic clone was characterized by both *cblA* gene sequences and a similar chromosomal fingerprint. In the current study we found that the *cblA* pilin subunit gene was present in 100% (38 isolates from 18 patients) of the epidemic ET12 isolates in our collection of *B. cepacia* strains. However, it was also found in one ET13 isolate from an adult CF patient living in a province which has never been particularly associated with epidemics of any nature. Since Sun et al. (36) also identified a single unrelated isolate with a divergent *cblA* NA sequence, the PCR amplicon from our ET13 isolate was sequenced and was found to be identical to that characterized for the epidemic strain. In view of this evidence it would appear that, upon a wider sampling, the *cblA* pilin subunit gene may prove to be more invariant among unrelated strains than reported by Sun et al. (36).

In addition to the *cblA* pilin subunit gene association, Sun et al. (36) also presented limited ribotyping data and phylogenetic analysis which appeared to support the clonal nature of these strains. In their investigation, two very similar ribotype patterns were identified for the epidemic strains, and it was suggested that this general profile could also be used to identify these strains. However, in our earlier work (13), in addition to the two RT patterns identified by Sun et al. (36), we identified five other RT patterns within the epidemic strains using an identical methodology. It should also be noted that the group of bands which were invariant among the ET12 isolates were also seen in other unrelated isolates of various ETs. With this in mind it would appear that the claims made by Sun et al. (36) as to the usefulness of ribotyping as a screening method were somewhat premature and perhaps overly optimistic.

In view of the potential impact on future protocols for the management of *B. cepacia*-colonized CF patients, caution is advised in the screening of isolates solely on the basis of markers attributed to a single highly transmissible strain. It is clear that the *cblA* pilin subunit gene, the ET12 genotype, and the IS402-IS1356 element appear to correlate well with this one particular epidemic strain, and the ability to identify this clone is not without value. However, as Sun et al. (36) also acknowledge, other distinct and highly transmissible epidemic lineages for which no genetic marker is currently available appear to exist. If any of these methods were prematurely implemented to screen CF patients, we would risk placing patients in the wrong groups, and the consequences in the case of these CF patients would be clinically and personally tragic.

The genome of *B. cepacia* is exceedingly complex, having three large chromosomal elements and a large plasmid, an arrangement apparently unique among eubacteria (24), and more genes involved in metabolism and pathogenicity need to be identified in order to provide important information on the virulence of clinical isolates. In view of the current data, it would appear that several genetic loci may need to be characterized and to agree in order to accurately assign clinical isolates to epidemic lineages. Until more is known about the pathogenicity of *B. cepacia* and the resulting implications to the CF community, we recommend that caution be applied before establishing any definitive phenotypic or genotypic screening criteria.

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Identification and Characterization of a Novel DNA Marker Associated with Epidemic *Burkholderia cepacia* Strains Recovered from Patients with Cystic Fibrosis

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Burkholderia cepacia is a problematic pathogen that may spread among patients with cystic fibrosis (CF). One highly infectious CF strain that causes epidemics in both the United Kingdom and eastern Canada has been shown to possess both the cable pilin subunit gene (*cblA*) and a unique combination of insertion sequences. However, no genetic markers linking this strain type with other types epidemic at various centers have been identified. Using a randomly amplified polymorphic DNA (RAPD) typing scheme, we identified an apparently conserved 1.4-kb fragment in the DNA fingerprint of epidemic *B. cepacia* strains. Conservation of the DNA marker among epidemic strains was demonstrated by Southern hybridization, and its prevalence was assessed in a collection of chromosomal DNAs extracted from 627 isolates representative of 132 RAPD-defined *B. cepacia* strain types. The marker was specifically associated with seven epidemic CF strains, was absent among nonepidemic strains infecting individual patients with CF, and rare among strains recovered from the natural environment. Only one of the seven epidemic CF strain types possessed DNA homologous to *cblA*. The RAPD marker was designated the "*B. cepacia* epidemic strain marker" (BCESM). Sequence analysis of chromosomal DNA corresponding to the 1.4-kb RAPD marker revealed the presence of a putative open reading frame (ORF) with significant homology to several negative transcriptional regulators; the ORF was designated the "epidemic strain marker regulator," or *esmR*. The BCESM DNA is the first genetic marker that has been identified to be specifically associated with and conserved among several epidemic *B. cepacia* strains which infect multiple patients with CF.

Despite its evolving role in pulmonary infection in patients with cystic fibrosis (CF), very little is known about the pathogenesis of *Burkholderia cepacia*. Colonization of CF patients with *B. cepacia* has serious clinical implications since the organism is highly virulent in certain patients (30) and is resistant to multiple antibiotics (4) and because patient-to-patient spread of the bacterium, first documented in 1990 (17), may occur. Evidence of the spread of *B. cepacia* strains among patients with CF and clustering of strain types at treatment centers has now been reported by a number of investigators (11, 18, 20); however, the factors which facilitate patient-to-patient transmission of *B. cepacia* remain poorly understood. Transmission of *B. cepacia* among CF patients may be dependent on a number of risk factors including strain type (29, 31), patient behavior and population (11), use of contaminated therapeutic devices (15), and CF treatment center practices (14).

Few *B. cepacia* virulence factors have been characterized phenotypically or studied at the genetic level, and little is known about their role in pathogenesis during CF infection. *B. cepacia* may bind to and colonize respiratory epithelia in CF patients by mechanisms similar to those described for *Pseudomonas aeruginosa* (6). Both organisms are generally motile and piliated and may adhere to the same disaccharide moiety present in many asialoglycolipids (21). *B. cepacia* also secretes a number of extracellular virulence factors including sid-

erophores, proteinases, hemolysins, and lipase (21). Evasion of the immune system may also be enhanced by the intrinsic resistance of *B. cepacia* to nonoxidative killing (27). *B. cepacia* recovered from CF patients whose clinical condition has undergone rapid deterioration binds to respiratory mucins with high affinity in vitro (23), and adherence to buccal epithelial cells may occur via both pilus-mediated and non-pilus-mediated adhesive mechanisms (24). To date, the only genetically characterized virulence factor associated with an epidemic *B. cepacia* strain type from CF patients is the cable pilus (10, 25, 29). Tyler et al. (31) recently described a novel insertion sequence (IS), IS1356, which, in association with the element IS402, may also serve as a genetic marker for the epidemic *B. cepacia* strain type with a cable pilus; the pathogenic significance of these ISs remains to be determined (31). These observations suggest that spread of the organism may also be linked to strain type; however, no genetic markers linking the *cblA*⁺ strain type and CF patient-derived strains epidemic at other treatment centers (18, 20) have been identified.

Using a PCR-based randomly amplified polymorphic DNA (RAPD) typing scheme, we typed 627 *B. cepacia* isolates recovered from CF patients and a variety of other sources (20). During the latter study, a DNA band with a conserved size was observed in the RAPD fingerprints of *B. cepacia* strain types that were epidemic (infecting multiple CF patients at certain treatment centers) and that were presumed to be transmissible. The amplified band was absent from the RAPD fingerprints obtained from nonepidemic *B. cepacia* strain types which infected individual CF patients and rarely occurred in fingerprints for isolates recovered from the environment. The marker was designated the "*B. cepacia* epidemic strain

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marker" (BCESM) because of its association with *B. cepacia* strain types infecting multiple CF patients. In this report, we describe the identification of this novel *B. cepacia* DNA marker by RAPD analysis, the prevalence of homologous DNA among a collection of 627 *B. cepacia* isolates, and sequence analysis of the BCESM DNA. The prevalence of the cable pilin subunit gene (*cblA*) (25) among the same collection of isolates is also presented. Despite the widespread use of RAPD fingerprinting for typing various microorganisms, very few of the arbitrarily amplified DNA markers have been studied. This report provides the first characterization of a RAPD marker specifically amplified from epidemic strains of *B. cepacia*.

MATERIALS AND METHODS

Bacterial isolation, strains, and culture. *B. cepacia* isolates recovered from patients with CF, patients without CF, and the environment were received from the contributors acknowledged previously (20). Microbiological culture, identification, and storage of isolates were carried out as described previously (20). *B. cepacia* C5424 (DNA isolation number B57), from which the BCESM was cloned (see below), was isolated from a CF patient in Vancouver, British Columbia, Canada; the RAPD type of the strain was type 2, and it was a member of the *cblA*⁺ (see below) major epidemic CF lineage (20, 25). *Escherichia coli* DH5aF⁺ was used to subclone *B. cepacia* DNA.

Preparation of bacterial DNA and RAPD analysis. For RAPD reactions and dot blot hybridization, genomic DNAs were extracted from the *B. cepacia* strains after mechanical disruption exactly as described previously (19, 20). RAPD fingerprinting with primer 272 (5'-AGCGGGCCAA-3') was performed as described previously (20). For restriction fragment length polymorphism (RFLP) analysis and subcloning, genomic DNAs were purified from the *B. cepacia* isolates as follows. Overnight bacterial growths from 2-ml Luria-Bertani broth cultures (grown with end-over-end rotation at 37°C in 13-ml screw-cap plastic tubes) were harvested by centrifugation. Bacterial pellets were resuspended in 200 µl of GET (50 mM glucose, 70 mM EDTA, 50 mM Tris-HCl [pH 8]) and 2.8 ml of lysis buffer (1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 8], 50 mM EDTA [pH 8]) containing 60 µg of RNase A per ml and 30 µg of proteinase K (Boehringer Mannheim, Laval, Quebec, Canada) per ml. The resulting lysate was incubated at 37°C with end-over-end rotation for 2 to 18 h prior to the addition of 1 ml of saturated ammonium acetate. Following vigorous mixing, protein and polysaccharide contaminants in the lysate were allowed to precipitate for 1 h at room temperature and were then removed by centrifugation (17,000 × g, 10 min). DNA was then collected from the lysate by ethanol precipitation (the addition of 2.5 volumes of ethanol), washed with 70% ethanol, dried under vacuum, and dissolved in 200 µl of TE (10 mM Tris-HCl [pH 8], 1 mM EDTA). The yield of DNA was approximately 100 µg per 2 ml of overnight bacterial growth.

Purification, labelling, and hybridization with the RAPD-derived BCESM probe. The 1.4-kb BCESM band amplified from *B. cepacia* C5424 (DNA number B57) with primer 272 by RAPD analysis (see Results) was separated from other DNA in the RAPD fingerprint by agarose gel electrophoresis (26); the DNA was then purified from an excised agarose slice with a glass bead DNA binding kit (Prep-A-Gene; Bio-Rad Laboratories, Mississauga, Ontario, Canada). RAPD fingerprints and genomic DNAs were probed with a 1-kb internal fragment of the 1.4-kb RAPD marker generated by endonuclease digestion with the enzyme *Pst*I. This internal probe was purified by agarose gel electrophoresis as described above, and 25 ng was labelled with 50 µCi of [³²P]dGTP (Amersham, Oakville, Ontario, Canada) with a randomly primed DNA labelling kit (Boehringer Mannheim, Laval, Quebec, Canada).

DNA amplified by RAPD analysis and endonuclease-digested *B. cepacia* genomic DNA were separated by agarose gel electrophoresis and transferred onto Hybond N⁺ nylon membranes (Amersham, Oakville, Ontario, Canada) by using conventional capillary transfer in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (26). The DNA was fixed on the membranes by exposure to UV irradiation according to the manufacturer's instructions, and the filters were prehybridized overnight at 65°C in the following hybridization buffer: 6× SSC, 0.5% SDS, 5× Denhardt's solution, and 100 µg of denatured fragmented salmon sperm DNA per ml (26). Denatured radiolabelled probe was added to fresh hybridization buffer, and hybridization was continued overnight at the same temperature. The filters were then washed at high stringency as follows: twice for 10 min at 65°C in 200 ml of 2× SSC-0.1% SDS, followed by two more washes in 200 ml of 0.1× SSC-0.1% SDS at the same temperature. Autoradiographs of the filters were obtained by exposure to X-ray film for 24 to 48 h.

Subcloning of RAPD marker and design of the BCESM PCR probes. The 1.4-kb RAPD marker from *B. cepacia* C5424 was purified as described above and was subcloned into the PCR product cloning vector pGEM-T (Promega, Fischer Scientific, Ottawa, Ontario, Canada). Cloning of the RAPD marker was confirmed by restriction endonuclease digestion with *Pst*I, and the resulting plasmid was designated pGEM-BC57. DNA sequence analysis of the ends of the BCESM

RAPD-PCR fragment was performed by dideoxy-termination PCR sequencing (CircumVent Thermal Cycle DNA sequencing kit; New England Biolabs, Mississauga, Ontario, Canada) by using the M13 forward and reverse primers. DNA sequences were analyzed manually on acrylamide gels (26).

Specific 18-base PCR primers able to amplify the 1.4-kb BCESM band were designed from the sequence internal to that of RAPD primer 272 (see Results and Fig. 4). The sequences of the BCESM-specific primers were as follows (5' to 3'): BCESM 1, CCACGGACGTGACTAACA, and BCESM 2, CGTCCATCCGAACACGAT. PCR mixtures (25 µl) containing 100 pmol of each primer, 20 ng of *B. cepacia* DNA, 250 µM (each) deoxynucleoside triphosphate, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, and 1 U of *Taq* polymerase (Canadian Life Technologies, Burlington, Ontario, Canada) were amplified on a Perkin-Elmer Cetus thermal cycler (model TC-1), as follows: 30 cycles of 1 min at 94°C, 1 min at 63°C, and 2 min at 72°C, followed by a final extension step at 72°C for 10 min. PCR products were analyzed by electrophoresis in 1.5% agarose gels as described previously (26).

Dot blot hybridization of *B. cepacia* DNA with the PCR-derived BCESM probe. For dot blot hybridization analysis, approximately 1 µg of DNA (dissolved in TE) from each of the 627 *B. cepacia* isolates was filtered under vacuum onto positively charged nylon membranes (Boehringer Mannheim) by using a 96-well dot-blot apparatus (Bio-Rad Laboratories). The BCESM DNA was amplified from *B. cepacia* C5424 by PCR with the specific primers described above and was simultaneously labelled with digoxigenin-11-uridine-5'-triphosphate (DIG-dUTP) according to the manufacturer's instructions (2a). Dot blot filters were hybridized with the DIG-labelled BCESM DNA under the same conditions described above for the radiolabelled probe. After stringent washing, the filters were developed by chemiluminescence detection with Lumi-Phos 530 according to the manufacturer's instructions (2a). After reaction with the BCESM probe, the filters were stripped of the probe by boiling them in 0.4 M NaOH for 10 min, and the filters were washed thoroughly with 2× SSC prior to hybridization with the cable pilus gene probe (see below).

Dot blot hybridization of *B. cepacia* DNA with the cable pilin subunit gene probe. A full-length cable pilin subunit gene probe was amplified by PCR from *B. cepacia* C5424 by using the primers and conditions described by Sajjan et al. (25). The 722-bp product was labelled during PCR with DIG-dUTP as described above. Prehybridization, hybridization, washing, and detection of the filters were carried out at high stringency exactly as described above for the BCESM probes.

Subcloning and sequence analysis of *B. cepacia* chromosomal BCESM region. Southern hybridization analysis of *B. cepacia* C5424 DNA localized the BCESM DNA to a 6.0-kb fragment that was generated by digestion of C5424 DNA with *Eco*RI and *Bam*HI. Digested chromosomal DNA in this size range was purified following agarose gel electrophoresis as described above and was subcloned into *E. coli* DH5aF⁺ by using *Eco*RI-*Bam*HI-digested pUC18 (26). The resultant recombinant clones (approximately 2,000 colonies) were divided into six pools and were grown briefly, and then plasmid DNA was extracted by alkaline lysis (26). Plasmid DNA pools were screened by PCR with the BCESM primers as described above. The BCESM DNA was amplified from two of the six pools, and one of these pools was retransformed into *E. coli*. PCR with the BCESM primers was then performed directly with individual colonies from this transformation, and two plasmid clones carrying the BCESM *Eco*RI-*Bam*HI region were identified in the first 36 colonies screened. One of these plasmids was designated pTF1.2 and was used in all subsequent characterizations of the BCESM region.

After restriction mapping of the insert of pTF1.2, a 3.3-kb *Eco*RI-*Sal*I fragment encoding the BCESM region was subcloned into pBluescript II SK⁺ (Stratagene, La Jolla, Calif.), generating the plasmid pTF11. A series of unidirectional nested deletions of the insert of this plasmid were created using the Erase-a-Base System kit (Promega, Madison, Wis.). Fifteen of the resulting deletion clones were sequenced by automated PCR sequencing (Applied Biosystems 377 Automated DNA Sequencer and AmpliTaq DyeDeoxy Terminator Cycle Sequencing) with the T7 primer. Sequence data were assembled and analyzed by computer software (Lasergene for Windows; DNASTAR Inc., Madison, Wis.).

Sequence homology analysis. Computer-assisted searches of the GenBank, PIR, and SWISSPROT databases were performed by using either the BLAST or the BEAUTY program (1, 32). Analysis of the putative EsmR protein was performed with the BLOCKS program (13).

Nucleotide sequence accession number. The BCESM DNA sequence has been submitted to GenBank and assigned GenBank accession number U81966.

RESULTS

Identification of a RAPD marker of conserved size in epidemic *B. cepacia* strain types. In a separate study we reported a RAPD method that was able to discriminate *B. cepacia* isolates recovered from a variety of sources including CF infection (20). Primary typing of *B. cepacia* was performed by RAPD analysis with primer 270; however, two additional RAPD primers, primers 208 and 272, were used to confirm the assignment of a given type (20). The RAPD profiles amplified by primer 272 from strains representative of the first 14 RAPD

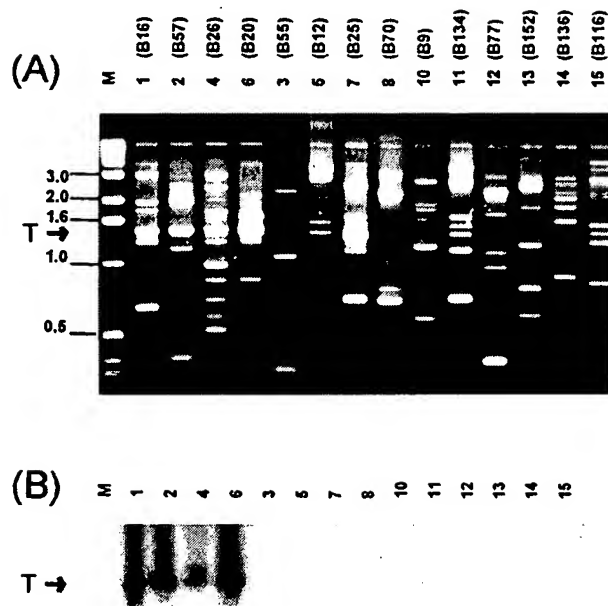


FIG. 1. RAPD fingerprints of *B. cepacia* isolates amplified with primer 272 and identification of the 1.4-kb marker. (A) DNA polymorphisms amplified from strains representative of 14 *B. cepacia* RAPD types (the RAPD type and strain number are indicated above each lane). The conserved 1.4-kb marker in RAPD types 1, 2, 4, and 6 is indicated by the letter T. Molecular size markers were run in lane M, and the sizes are indicated in kilobases. (B) Southern blot of the DNA in panel A probed with a 1.0-kb *Pst*I fragment derived from the 1.4-kb marker purified from strain C5424 (B57). The position of the hybridizing marker is indicated by the letter T.

types are shown in Fig. 1A. The fingerprint profiles of each strain type obtained with primer 272 were distinct, discriminated between strain types, and correlated with typing groups established with primer 270. However, a 1.4-kb DNA band amplified by primer 272 appeared to be conserved in the fingerprints of *B. cepacia* strain types for which there was evidence of patient-to-patient spread among CF patients in Vancouver (types 1, 2, 4, and 6; Fig. 1A) and absent from the other nine nonepidemic CF strain types and one environmental strain type (Fig. 1A).

B. cepacia type 1 was the predominant strain type colonizing 9 CF patients attending the pediatric clinic in Vancouver, type 2 was the epidemic strain type affecting more than 120 patients at treatment centers in the United Kingdom and across Canada, type 4 was the predominant strain type among patients attending the adult CF clinic in Vancouver, and type 6 *B. cepacia* infected a total of 5 pediatric CF patients in Vancouver (20). Types 3, 5, 7, 8, 10, 12, and 14 (Fig. 1A) were each isolated from individual CF patients only (20); *B. cepacia* type 15 stably colonized one CF patient and was cultured on only one occasion from another CF patient who later became stably colonized with a type 6 strain (20). Type 13 isolates were recovered from three CF patients in Manchester, United Kingdom (20); however, the 1.4-kb marker was not apparent in the fingerprint profile in Fig. 1A of the representative isolate amplified by RAPD analysis. Type 11 *B. cepacia* was isolated from soil (20). Although the 1.4-kb marker was conserved in size among the majority of isolate types which infected multiple CF patients, because of the arbitrary nature of RAPD analyses, it

may not have been homologous among the different strain types.

Conservation of the *B. cepacia* epidemic strain marker. Conservation of the BCESM DNA was demonstrated by the following experiments. The 1.4-kb DNA fragment was purified from the RAPD fingerprints of type 1, 2, 4, and 6 *B. cepacia* strains, and endonuclease cleavage of the DNA with *Hae*III and *Pst*I revealed identical RFLP profiles (data not shown). Conservation of the RFLP profile suggested that the chromosomal region amplified by primer 272 in these epidemic *B. cepacia* strain types was homologous. The absence of the 1.4-kb marker from the nonepidemic CF isolates and the environmental isolate examined (Fig. 1A) suggested that this chromosomal region was either missing or rearranged such that amplification by PCR was not possible. To further assess this chromosomal locus, both the randomly amplified DNA and the chromosomal DNA from *B. cepacia* were probed by Southern hybridization for the presence of sequences homologous to the 1.4-kb RAPD marker.

Southern hybridization analysis of the RAPD gel shown in Fig. 1A with a probe derived from the 1.4-kb RAPD marker of *B. cepacia* C5424 is shown in Fig. 1B. The 1.4-kb bands from all the *B. cepacia* isolates representative of the epidemic strain types except for type 13 (see below) hybridized to the probe (types 1, 2, 4, and 6); homologous DNA was not present in the RAPD fingerprints of the other strain types in Fig. 1A, indicating that the marker band was not amplified, even at a different size range, for these isolates. Southern hybridization of genomic DNA digested with *Pst*I also revealed the presence of homologous 1-kb DNA in epidemic strain types 1, 2, 4, and 6 (data not shown); no homologous DNA at any size range was detected in the nonepidemic strain types examined (data not shown). Examination of restriction enzyme-digested genomic DNA from *B. cepacia* C5424 by Southern hybridization localized the marker DNA to a 6-kb *Eco*RI-*Bam*HI chromosomal fragment (data not shown). Because the 1.4-kb RAPD fragment had been localized to a chromosomal locus and was truly absent from the nonepidemic CF isolates examined, the marker was designated BCESM and was cloned in *E. coli* for further characterization (see below).

Subcloning of BCESM RAPD band and design of specific PCR primers. In order to develop a specific PCR probe for the BCESM region and to subsequently facilitate cloning of the chromosomally encoded marker region, the 1.4-kb RAPD-PCR marker amplified from strain C5424 was cloned in *E. coli*, generating plasmid pBC57 (see Materials and Methods). Sequence analysis of each end of the cloned 1.4-kb PCR fragment enabled the design of specific PCR primers by using the sequences internal to that of RAPD primer 272 which formed the ends of the amplified marker (see Fig. 4). These specific 18-base PCR primers, BCESM 1 and BCESM 2 (see Materials and Methods), enabled the 1.4-kb marker to be amplified from the chromosomal DNAs of positive *B. cepacia* strains (Fig. 2).

Amplification of the BCESM by RAPD analysis from strains of types 1, 2, 4, and 6 had been demonstrated by Southern hybridization (Fig. 1), and these strain types were also positive for the specific PCR marker (Fig. 2). Type 13 isolates, which lacked the marker by RAPD analysis, but which had the characteristics of an epidemic *B. cepacia* strain, were found to amplify a single fragment by specific PCR (Fig. 2). The size of the marker amplified from type 13 isolates was slightly higher than that of the other epidemic types; however, the RFLP profile generated by digestion with *Hae*III was conserved (Fig. 2) except for the largest cleavage product, suggesting that the BCESM of type 13 strains may contain minor DNA sequence variations in this region. Type 17 isolates, the predominant CF

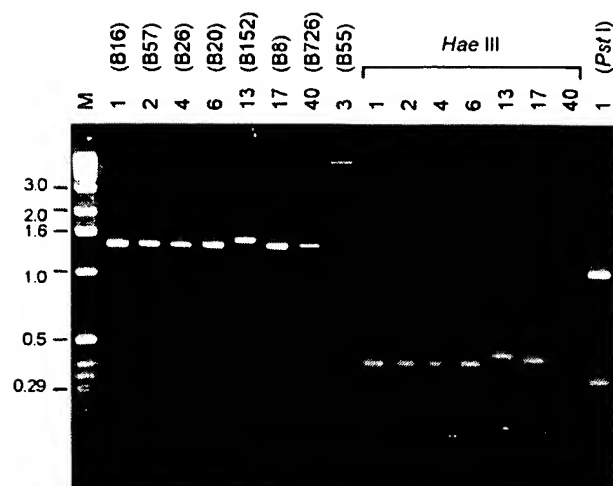


FIG. 2. Specific amplification of the BCESM DNA from transmissible *B. cepacia* strain types by PCR. The 1.4-kb BCESM DNA was amplified with the specific primers BCESM 1 and BCESM 2 and the conditions described in the Materials and Methods. One-tenth of each PCR mixture was loaded for types 1, 2, 4, 13, 17, and 40 (RAPD type and strain DNA number are indicated above each lane); half of the PCR mixture was loaded for the negative control type 3 (chromosomal template DNA forms the band at the top of this lane). The products obtained after *Hae*III digestion of the BCESM band amplified from each type and the *Pst*I digestion products of the marker from a type 1 strain (B16) are indicated on the right. Molecular size markers are shown in lane M, and the sizes are indicated in kilobases.

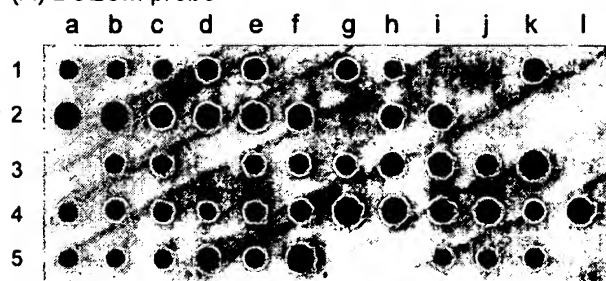
strain type at a treatment center in Cleveland, Ohio (18), and type 40, an epidemic *B. cepacia* CF strain from a center in Australia (20), were also positive for the marker (Fig. 2). No product was observed after amplification of DNA from the nonepidemic CF isolates (amplification of the DNA of a type 3 isolate is shown in Fig. 2). The BCESM DNA amplified by specific PCR shared the same *Hae*III-derived and *Pst*I-derived RFLP pattern as the RAPD marker (Fig. 2) and also hybridized to the RAPD-derived fragment (data not shown), demonstrating that both types of probe detected the same chromosomal sequence.

The specific PCR primers also facilitated the cloning of chromosomal BCESM DNA from *B. cepacia* C5424 into *E. coli* with the vector pUC18 (26) (see Materials and Methods).

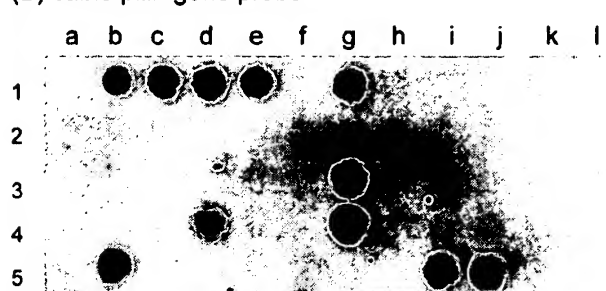
Prevalence of BCESM DNA among *B. cepacia* isolates recovered from a variety of sources. To assess the prevalence of the BCESM region among *B. cepacia* isolates, total genomic DNA from 627 strains, previously assessed by RAPD analysis (20), was examined by DNA dot blot hybridization with the specific PCR-derived BCESM probe from strain C5424 (Fig. 2, DNA sample B57); the results are summarized in Table 1. Hybridization was carried out at high stringency in order to identify sequences homologous to the BCESM probe (see Materials and Methods), and the signals obtained from the DNAs of control strains known to be positive for the marker were strong (Fig. 3, DNA samples B16, B20, B26, and B57). The BCESM PCR probe hybridized with the DNAs of all 128 CF isolates which belonged to the epidemic RAPD types 1, 4, 6, and 13. Of the three other *B. cepacia* types which infected multiple CF patients (types 2, 17, and 40), DNAs from a total of five *B. cepacia* isolates belonging to these fingerprint types did not hybridize to the marker (Table 1); two type 2, one type 17, and two type 40 isolates were negative for BCESM DNA (Table 1).

Of the remaining CF isolates examined, 101 were recovered from a total of 57 individual CF patients and were not shared

(A) BCESM probe



(B) cable pilin gene probe



(C) Strain DNA number

	a	b	c	d	e	f	g	h	i	j	k	l
1	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
2	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22	B23	B24
3	B25	B26	B27	B28	B29	B30	B31	B32	B33	B34	B35	B36
4	B37	B38	B39	B40	B41	B42	B43	B44	B45	B46	B47	B48
5	B49	B50	B51	B52	B53	B54	B55	B56	B57	B58	B59	B60

FIG. 3. Dot blot hybridization of *B. cepacia* DNA. The autoradiographs obtained after hybridization of DNA from the first 60 *B. cepacia* isolates with the BCESM PCR probe (A) and the cable pilin probe (B) are shown. The strain DNA number is indicated in panel C. Probe labelling and hybridization were as described in the Materials and Methods.

by another patient (nonepidemic CF patient-derived and unique CF patient-derived types; Table 1) (20). Three of the types classified as nonepidemic were recovered from two CF patients. Infection of the two CF patients with type 15 *B. cepacia* isolates was transient (20). Type 23 and type 35 *B. cepacia* isolates were each recovered from two CF patients at treatment centers in Oklahoma City and Edinburgh, United Kingdom, respectively; no further isolates of these types were found in the collection to support their categorization as epidemic (20). Among the isolates recovered from patients without CF, 20 of 44 isolates (45%) possessed homologous DNA (Table 1). Only 5 of the 58 isolates (8.6%) recovered from environmental sources harbored DNA homologous to the BCESM probe (Table 1).

Total genomic DNAs from the following organisms did not hybridize to the BCESM probe (*n* indicates the number of strains tested): *Agrobacterium radiobacter* (*n* = 1), *Alcaligenes faecalis* (*n* = 1), *Alcaligenes xylosoxidans* (*n* = 5), *Burkholderia gladioli* (*n* = 13), *Candida* spp. (*n* = 1), *Comamonas acidovorans* (*n* = 5), *Enterobacter agglomerans* (*n* = 1), *E. coli*

TABLE 1. Hybridization of the BCESM probe to DNA isolated from *B. cepacia* isolates recovered from a variety of sources

Source and RAPD type ^a	No. of isolates	No. of isolates with the following hybridization result:		% Prevalence of positive strains
		Positive	Negative	
Epidemic CF RAPD types (no. of patients colonized) ^b				
1 (15)	48	48	0	100
2 (>119)	267	265	2	99
4 (21)	52	52	0	100
6 (5)	23	23	0	100
13 (3)	5	5	0	100
17 ^c (>2)	5	4	1	80
40 (17)	17	15	2	88
Nonepidemic CF RAPD types ^d 3, 5, 7, 8, 10, 12, 14, 15, 19, 23, 24, 33, and 35				
	50	0	50	0
Unique CF isolates ^e				
	58	8	50	16
Clinical ^f RAPD types 1, 4, 9, 29, 32, 36, 38, and 39				
	28	13	15	46
Unique clinical isolates ^e				
	16	7	9	43
Environmental ^g RAPD types 2, 11, 14, 18, 21, 22, 24, 25, 26, 28, 30, 31, 34, 37, and 39				
	37	3	34	6
Unique environmental isolates ^e				
	21	2	19	9
All isolates				
CF isolates	525	420	105	80
Clinical isolates	44	20	24	45
Environmental isolates	58	5	53	12
Total	627	445	182	71

^a Adapted from RAPD typing data described by Mahenthiralingam et al. (20).^b RAPD type infecting more than three CF patients.^c Type 17 isolates were the dominant strain ribotype among patients attending a Cleveland CF treatment center (18); however, the 5 isolates present in our collection were representative of isolates recovered from only two of these patients.^d RAPD type infecting individual CF patients at the time of data collection and isolate culture.^e Strains with unique RAPD fingerprints unmatched in our collection at the time of data collection.^f *B. cepacia* strains isolated from patients without CF.^g *B. cepacia* strains recovered from the environment.

(*n* = 2), *Chryseobacterium* (*Flavobacterium*) *meningosepticum* (*n* = 1), *P. aeruginosa* (*n* = 15), *Mycobacterium tuberculosis* (*n* = 2), and *Stenotrophomonas* (*Xanthomonas*) *maltophilia* (*n* = 4) (data not shown).

Correlation of the BCESM marker and *B. cepacia* RAPD type. Since the majority of CF isolates in our collection belonged to the epidemic CF RAPD types 1, 2, 4, 6, 13, 17, and 40 (417 of 525 CF isolates; Table 1), the bias in total numbers

may have skewed the distribution of the BCESM probe. However, because all 627 isolates had been typed by RAPD analysis, the distribution of the BCESM DNA was also examined by strain type; these data are summarized in Table 2.

In total, 78 distinct RAPD types of *B. cepacia* were recovered from CF patients, and 15 of these possessed BCESM DNA (Table 2). Of the positive strain types, seven types were epidemic and infected multiple CF patients, as explained

TABLE 2. Prevalence of BCESM DNA and *cblA* DNA among *B. cepacia* RAPD strain types

RAPD type ^a	Total no. of strains	No. BCESM positive (RAPD type)	No. <i>cblA</i> positive (RAPD type)
CF epidemic type	7	7 (1, 2, 4, 6, 13, 17, and 40)	1 (2)
CF nonepidemic type	13	0	0
CF unique type	58	8	1
Clinical type ^{b,c}	8	3 (1, 4, and 38)	0
Clinical unique type	16	4	0
Environmental type ^{c,d}	15	2 (2 and 26)	2 (2 and 31)
Environmental unique type	21	2	3
Total ^e	132	23 [17.4%]	6 [4.5%]

^a Adapted from RAPD typing data described by Mahenthiralingam et al. (20).^b Both clinical and CF isolates of RAPD types 1 and 4 were included in this category.^c Both clinical and environmental isolates of RAPD type 39 were included in this category.^d Both CF and environmental isolates of RAPD types 2, 14, and 24 were included in this category.^e The totals were calculated after subtraction of the six strain types recovered from more than one source, which are described in footnotes b, c, and d.

above; the eight remaining positive types had a unique fingerprint, and each was recovered from an individual CF patient in disparate geographical locations (20). Therefore, in contrast to the data obtained from the total number of isolates, the majority of CF strain types lacked BCESM DNA. Sixty of the negative CF strain types (10 nonepidemic CF types and 50 unique CF types; Table 2) were each recovered from individual CF patients, and no evidence of spread of these strain types was apparent. Of the three remaining BCESM-negative CF strain types (types 15, 23, and 35), evidence of patient-to-patient spread was not substantiated by further data (20).

Among the *B. cepacia* strain types recovered from patients without CF, 7 of a total of 24 types possessed the marker (Table 2). Of the 36 strain types recovered from the environment, 4 possessed DNA homologous to the BCESM probe. Two of these BCESM-positive environmental strain types were isolated from hospital environments (types 2 and 26; Table 2) (20). The remaining two BCESM-positive types were recovered from the natural environment (20).

Prevalence of the cable pilus gene, *cblA*. The DNAs from all 627 *B. cepacia* isolates were also hybridized with the cable pilus gene probe (*cblA*) (25). Dot blot hybridization results for DNA from the first 60 isolates are presented in Fig. 3B. In total, 272 of the 627 isolates tested possessed DNA homologous to the *cblA* gene, and of these, 267 (98%) belonged to the same strain lineage, RAPD type 2, the major CF RAPD strain type (20) representative of this epidemic lineage (29, 31). None of the other epidemic CF strain types or nonepidemic CF strain types possessed DNA homologous to *cblA*. One CF isolate with a unique RAPD fingerprint, recovered from a patient in Oklahoma, hybridized with the *cblA* probe; DNA from this isolate did not hybridize with the BCESM probe (Table 2). The *cblA* gene was present in *B. cepacia* ATCC 35130, an environmental isolate, and another environmental isolate from Mexico which shared the same RAPD type (type 31) as ATCC 35130 (Table 2). Two further environmental isolates from distinct geographical sources (the United States and the United Kingdom) with unique RAPD fingerprints possessed cable pilus DNA; both of these lacked DNA homologous to the BCESM probe. None of the clinical isolate types recovered from patients without CF carried the cable pilus gene (Table 2). In total, only 6 of the 132 RAPD-defined *B. cepacia* strain types possessed DNA homologous to the cable pilin subunit gene (Table 2).

Nucleotide sequence of the BCESM DNA. A 6.0-kb chromosomal DNA fragment from *B. cepacia* C5424 (B57) encoding BCESM was cloned in *E. coli*; the nucleotide sequence from 3.4 kb of DNA encompassing the BCESM probe was determined (see Materials and Methods). The features of the 1,600 bp of DNA spanning the BCESM marker are shown in Fig. 4A, and the corresponding nucleotide sequence is shown in Fig. 4B.

Sequences identical to those of PCR primers BCESM 1 and BCESM 2 were found 1,419 bp apart, and recognition sites for the endonuclease *Pst*I, used to generate the first RAPD-derived 1.0-kb probe, were encoded 1,022 bp apart within the 1,419-bp PCR product (Fig. 4). The sequence of RAPD primer 272 (5'-AGCGGGCCAA-3'), which originally amplified the BCESM DNA, was only partially matched upstream and downstream of the sequences encoding the specific BCESM PCR primers (Fig. 4A). A total of 8 bp upstream and 9 bp downstream were matched at the 3' terminus of the primer binding site (Fig. 4B). Although this level of homology was sufficient for amplification of the marker from most of the epidemic strain types under the low-stringency RAPD-PCR conditions, the degree of mismatch may explain the inability of RAPD analysis to amplify the marker from type 13 strains,

which may contain further sequence differences in this region (Fig. 1 and 3).

Sequence homology analysis of the BCESM DNA. Sequence analysis of the marker revealed the presence of a 834-bp open reading frame (ORF) encoding a putative 277-amino-acid protein which was internal to the 1.4-kb BCESM marker (Fig. 4). Comparison of the sequence of this ORF with nucleotide and protein sequences present in the standard databases demonstrated significant homology to several negative transcriptional regulators. The putative ORF was designated the "epidemic strain marker regulator," or *esmR*, because of the homology to negative transcriptional regulators. The putative *EsmR* protein possessed the highest similarity (of 50 to 60% over several domains) with YhcK, a hypothetical transcriptional regulator from *E. coli*; UxuR, a regulator of glucuronate metabolism in *E. coli* (2, 3); PdhR, a negative transcriptional regulator of the pyruvate dehydrogenase complex of *E. coli* (28); and LldR, a putative regulator gene of *E. coli* involved in lactate dehydrogenase production (8). The region of highest similarity occurred in the N-terminal domains of all of the homologs, in which a helix-turn-helix domain was present. The putative *EsmR* protein also possessed a helix-turn-helix domain (Fig. 4B), suggesting that it is involved in a regulatory capacity in *B. cepacia*. Thus, *EsmR* likely belongs to the GntR family of bacterial regulatory proteins (12).

DISCUSSION

B. cepacia infection in patients with CF is problematic. Knowledge of the risk factors leading to transmission and the features of *B. cepacia* which may enable it to spread from one CF patient to another is limited. We have identified by RAPD analysis a novel region of DNA specifically associated with epidemic strain types of *B. cepacia* which infect multiple CF patients at various treatment centers. The identification of this marker by RAPD analysis illustrates the versatility of the PCR-based technique for the genomic characterization of organisms for which genetic knowledge is limited. Identification of a putative ORF within the marker DNA sequence, which shares homology with a family of negative transcriptional regulators, may provide insights into the virulence factors responsible for patient-to-patient spread, aid in the identification of problematic strain types, and assist in the diagnosis and management of *B. cepacia* infections in patients with CF.

To date, only the cable pilus and mesh pilus virulence factors have been linked with epidemically transmitted CF isolates of *B. cepacia* (10, 29). Goldstein et al. (10) categorized *B. cepacia* CF isolates as "epidemically transmitted" and "nonepidemically" or "low-level transmissibility" isolates from their epidemiological backgrounds. Strains possessing the cable and mesh pilus types were categorized with "epidemically transmitted" CF isolates (10). Such division of *B. cepacia* strains must be approached with caution since the risk factors for *B. cepacia* transmission and the pathogenesis of the organism are not fully understood. Tyler et al. (31) recently described IS markers which were also specifically associated with cable pilus-linked epidemic lineage. The BCESM DNA identified in this study is predominantly associated with strains of *B. cepacia* that infect multiple CF patients at various treatment centers and that by definition were epidemic at each center. However, since the available epidemiological data on the colonized patients was limited, assumptions on the exact mode and frequency of transmission of these epidemic *B. cepacia* strain types cannot be accurately made.

The *B. cepacia* epidemic strain types infecting multiple CF patients attending the Vancouver, Cleveland, Manchester, and

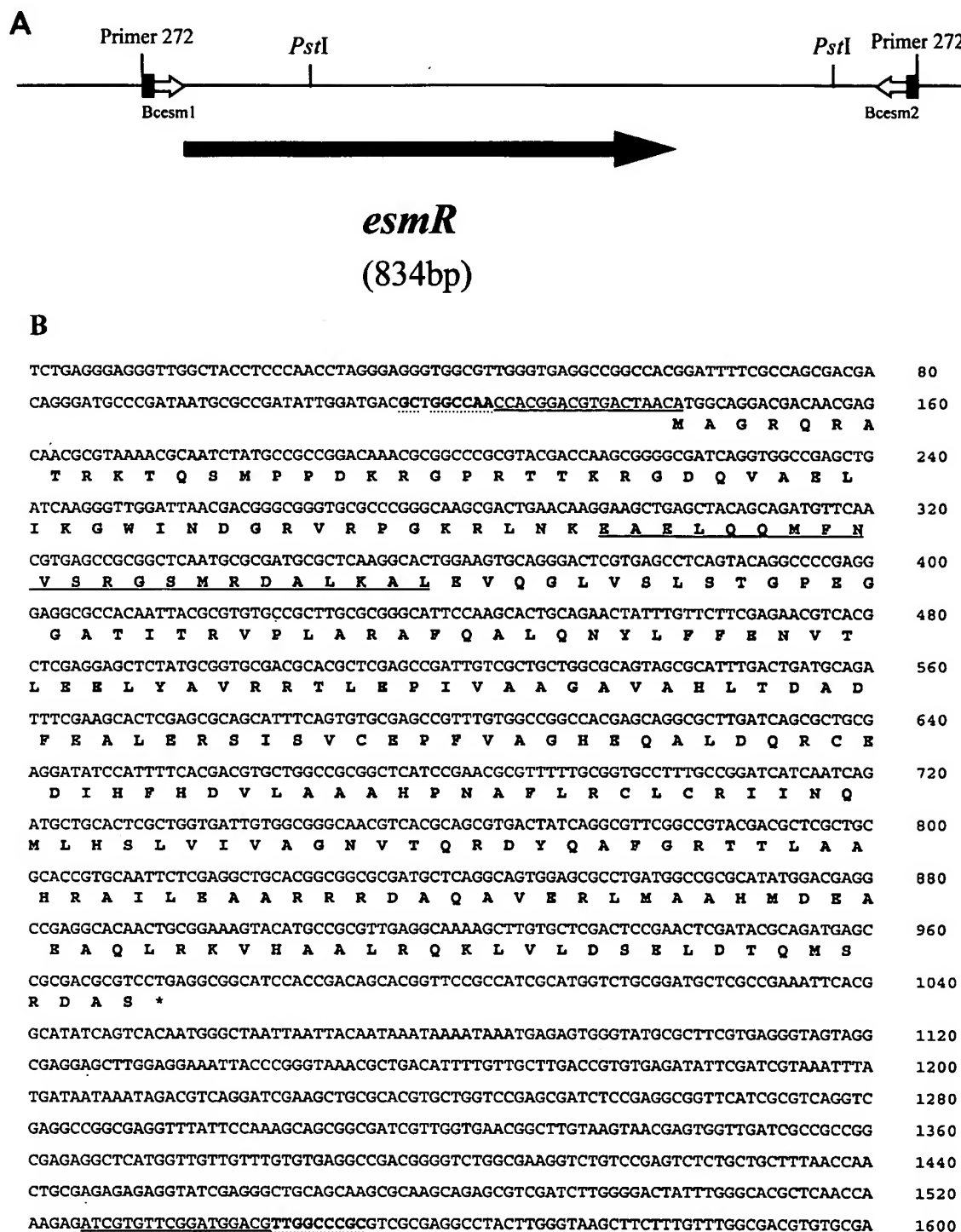


FIG. 4. BCESM chromosomal locus of *B. cepacia* C5424. (A) Features of 1,600 bp of the nucleotide sequence encompassing the marker. The RAPD primer binding sites, the BCESM primer binding sites, the *Pst*I cleavage sites, and the orientation of a putative ORF, *esmR*, are shown. (B) The corresponding 1,600-bp nucleotide sequence and amino acid translation of the putative ORF. Nucleotides matching RAPD primer 272 are indicated with a dotted line, and the sequences of the BCESM PCR primers are underlined. The amino acid translation of the putative EsmR protein is indicated by boldface type beneath the nucleotide sequence. The underlined region of EsmR corresponds to the putative helix-turn-helix domain homologous with the helix-turn-helix domains of a number of negative transcriptional regulators (12).

Australian treatment centers all lacked the cable pilus gene, but did possess DNA homologous to the BCESM (types 1, 4, 6, 13, 17, and 40; Table 1). The absence of the cable pilus marker in all the other epidemic strain types examined in this

study is in contrast to the results of Sun et al. (29), whose studies highlighted the spread of only one strain type among CF patients and promoted the fact that the *cblA* DNA is a useful marker for infectious strain types. Our data demonstrate

that all *B. cepacia* colonization among patients with CF is of major concern and that strain types other than those with the *cblA*⁺ lineage should not be considered less of a problem just because the cable pilus-carrying strain has been designated "highly infectious" (10, 30). Although the *cblA* marker is associated with the most prevalent *B. cepacia* CF strain type in the United Kingdom and North America (RAPD type 2; Tables 1 and 2) (20), it was present in only 6 of the 132 RAPD-defined strain types in the collection of 627 isolates examined (Table 2); of these 6 *cblA*⁺ types, only 1 was an epidemic CF strain type (type 2), as defined by our collection. Interestingly, *B. cepacia* RAPD type 2 was the only strain type in the entire collection possessing both the cable pilin subunit gene and the BCESM DNA. Since the combination of IS1356 and IS402 is also associated with this infectious strain type (31), these data suggest virulence factors linked with this unique combination of genetic markers may contribute to the striking prevalence of this lineage within the CF patient population.

DNA homologous to the BCESM probe was absent from 5 of 417 isolates belonging to epidemic CF types, suggesting that the region may be subject to some instability. Multiple replicons are present in the genome of *B. cepacia* (7, 22), and therefore, it is possible that the BCESM region may be encoded on a replicon which may be unstable in some transmissible CF isolates. There was no correlation with the BCESM marker and the presence of a plasmid when conventional alkaline lysis (26) was used as a means of DNA preparation (data not shown); however, this procedure is inefficient for the purification of plasmids greater than 50 kb in size (26). The genome of *B. cepacia* may also be quite plastic due to large numbers of ISs present (9), and specific ISs have recently been linked to the *cblA* epidemic strain type (31). No sequences homologous to IS elements present in databases were found on the sequence of the BCESM region; however, if IS elements flank the BCESM region that we have characterized, this may contribute to its instability in vitro in certain strain types.

The majority of CF strain types lacked the BCESM, and hence, the DNA cannot be considered a universal marker for the ability of *B. cepacia* to colonize and cause infection in patients with CF. However, the specific association of the BCESM DNA with strain types which infect multiple CF patients suggests that it can be used to identify strain types which have a high capacity to spread among CF patients and become epidemic at a given treatment center. For example, type 6 *B. cepacia*, a BCESM-positive strain, colonized only one CF patient at the treatment center in Vancouver for 6 years (20). In the seventh year of study, this strain type spread to two other pediatric CF patients and subsequently to more patients to infect a total of five CF patients attending the treatment center (20). Because of this specific association with strain transmission and not colonization per se, the BCESM DNA may mark a region of the *B. cepacia* genome which encodes virulence factors or regulators which play a role in strain transmissibility. The role of the putative EsmR protein encoded on the 1.4-kb BCESM DNA remains to be determined. Significant homology to the GntR family of bacterial regulatory proteins and possession of a DNA-binding helix-turn-helix motif (12) suggest that it may play a regulatory role in *B. cepacia*; however, the ORF's authenticity and regulatory targets remain to be determined.

Phenotypic examination of *B. cepacia* strains recovered from the natural environment has indicated that they are markedly different from epidemic strains associated with pulmonary infection in CF patients (5), and multilocus enzyme electrophoresis has also shown that environmental isolates produce electrophoretic profiles that are in general distinct from those

of CF isolates (16). The low prevalence of the BCESM DNA among *B. cepacia* strains recovered from the natural environment (2 of 36 types examined; Table 2) suggests that in general these strain types are also genetically distinct from the strains which infect multiple CF patients. Identification of the BCESM DNA region, which is the first genetic marker conserved among several epidemic types of *B. cepacia*, suggests that phenotypic factors which are associated with this DNA but which are absent from isolates present in the natural environment may be important for the spread of this organism among patients with CF.

The *B. cepacia* BCESM DNA did not hybridize to DNA from a wide variety of other microorganisms present in sputum, and the clear distinction between positive and negative *B. cepacia* strains suggests that DNA probes based on this region may be useful clinically for the identification of potentially problematic strain types. The prevalence of the *B. cepacia* BCESM marker will need to be evaluated in other CF treatment centers where transmissible isolates are thought to be present. However, on the basis of the results presented in this report, the BCESM probe is the first genetic link identified between several epidemic strain types, and it may serve as a putative marker for the presence of strains of *B. cepacia* with the potential for spread among patients with CF.

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REVIEW ARTICLE

***Burkholderia cepacia*: medical, taxonomic and ecological issues**

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The increasing challenge posed by multiresistant saprophytes in medical microbiology is strikingly demonstrated by the emergence of *Burkholderia* (formerly *Pseudomonas*) *cepacia* as an opportunist pathogen in immunocompromised patients, particularly individuals with chronic granulomatous disease and cystic fibrosis (CF). Best known previously as a phytopathogen and the cause of soft rot of onions, *B. cepacia* presents three major problems for the CF community: innate multiresistance to antimicrobial agents; person-to-person transmission of epidemic strains through nosocomial or social contacts; and 'cepacia syndrome', a fulminating fatal pneumonia, sometimes associated with septicaemia, that occurs in approximately 20% of colonised patients, including those with previously mild disease. Accumulated evidence to dispel earlier suggestions that the organism is avirulent and merely a marker of existing lung disease includes: case-controlled studies in CF patients; reports of serious infections in non-CF patients; in-vitro and in-vivo evidence that *B. cepacia* induces production of pro-inflammatory markers, including the major cytokine TNF α ; and histopathological evidence that exposure of transgenic CF mice to *B. cepacia* results in pneumonia. By the early 1990s, the use of selective culture media and DNA-based bacterial fingerprinting confirmed suspicions of epidemic person-to-person spread of *B. cepacia*. This evidence provided scientific justification for draconian and controversial measures for infection control, in particular, segregation of *B. cepacia*-colonised patients during treatment at CF centres and their exclusion from social gatherings and national conferences. Recently, molecular analyses of type strains and clinical isolates have revealed that isolates identified previously as *B. cepacia* belong to at least three distinct species and have increased concern regarding the reliability of current laboratory detection and identification systems. Clarification of the taxonomy of *B. cepacia*-like organisms and the pathogenic potential of environmental isolates remains a high priority, particularly when the organism's antifungal and degradative properties have created interest in its potential use as a biological control agent to improve crop yields and its use for the bioremediation of contaminated soils.

Introduction

'The development of multiresistance in major microbial pathogens is well-recognised; in contrast, little attention has been paid to the pathogenic potential of naturally resistant environmental saprophytes'.

Known originally as a phytopathogen, *Burkholderia cepacia* (previously *Pseudomonas cepacia*, *P. multi-*

vorans, *P. kingii*, 'Eugonic oxidiser 1') exhibits impressive nutritional versatility. Some microbes have an inherent or acquired ability to degrade antibiotics, but few have the ability to use penicillin as a sole carbon source [1] or to reduce onions to a macerated pulp! The earlier name, *P. multivorans*, reflected the organism's omnivorous appetite, but it was not until 1950 that its pathogenic potential was recognised when Burkholder identified the organism as the cause of soft rot of onions—particularly 'compromised' onions damaged during harvesting—and provided an appropriate species epithet (Latin: *cepia* = onion) [2]. In the early 1990s, following taxonomic re-appraisal, the RNA group II pseudomonads were recognised as

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the new genus *Burkholderia*, with *B. cepacia* as the type species [3]. At present, the genus *Burkholderia* comprises *B. cepacia*, *B. gladioli*, *B. mallei*, *B. pseudomallei*, *B. caryophylli*, and recently added to the group, *B. plantarii*, *B. glumae*, *B. vandii* [4], *B. coccovenans* [5] and *B. vietnamiensis* [6].

The general characteristics of *B. cepacia* include the following: gram-negative, non-spore-forming, aerobic bacillus; motile with a respiratory metabolism and typically catalase- and oxidase-positive; various non-fluorescent pigments may be produced and poly- β -hydroxyalkanoates can be accumulated as reserve materials; the optimal temperature for growth is 30–35°C [7]. Recently, elegant molecular analyses have provided scientific evidence that may account for the organism's impressive versatility, including multilocus linkage disequilibrium analysis of environmental populations [8]—which suggested an extraordinarily high rate of recombination in *B. cepacia* relative to binary fission—and demonstration of multiple replicons and insertion sequences in type strains [9, 10].

The natural habitats of *B. cepacia* have been described as soil, water and vegetation [11]. However, it is a common but erroneous belief that *B. cepacia* is a ubiquitous saprophyte sharing similar environmental habitats with *Pseudomonas aeruginosa* and other pseudomonads. Extensive surveillance studies have shown that culture of *B. cepacia* from natural sources, including soil, water and plants, or from hospitals, foodstores, restaurant salad bars and patients' homes is surprisingly difficult, with detection rates of only 1–16% [12–16].

In agricultural microbiology, ecological awareness and an increasing incidence of pesticide-resistant pathogens have led to interest in *B. cepacia* as a potential agent for biological control and soil decontamination. *B. cepacia* produces several antimicrobial agents, including pyrrolnitrins, altericidins, cepalymins and bacteriocin-like agents [17–20], that inhibit bacterial and fungal phytopathogens and suppress tobacco wilt and other plant diseases [21]. *B. cepacia* is also capable of degrading industrial waste and herbicides, including 2,4,5-trichlorophenoxyacetic acid (2,4,5-T),

the principal ingredient of the highly potent 'agent orange' [22]. Indeed, *B. cepacia* has been shown to degrade 2,4,5-T in heavily contaminated soils at a rate up to 20 000-fold greater than other known degradative bacteria [23].

In contrast to its potential agricultural benefits, *B. cepacia* has also emerged as a multiresistant opportunist human pathogen, leading to concern about the relationship between environmental and clinical isolates [14, 24–26] and the potential hazards of releasing *B. cepacia* as a biological control agent [14, 24]. This review will provide an update on microbes currently described as *B. cepacia*, with particular focus on clinical, taxonomic and ecological issues (Table 1) associated with pulmonary infection in patients with cystic fibrosis (CF).

The emergence of *B. cepacia* as a human pathogen

Before the early 1980s, reports of human infections caused by *B. cepacia* were sporadic and generally restricted to hospitalised patients exposed to contaminated disinfectant and anaesthetic solutions in which this nutritionally adaptable saprophyte survives for long periods. Infections included those of soft tissues and the respiratory and urinary tracts, but bacteraemia also occurred, sometimes associated with endocarditis and septic shock [27–31]. A rising incidence of *B. cepacia* infection was noted during the early 1980s and, although in some cases culture of *B. cepacia* was thought to reflect mere colonisation or contamination rather than infection [11, 32], extensive analyses of USA databases of nosocomial infections confirmed a significant increase in clinically significant *B. cepacia* infections [33, 34]. The apparent propensity of *B. cepacia* to cause fatal pulmonary infections, as suggested by these analyses, is emphasised in patients with chronic granulomatous disease (CGD)—in whom *B. cepacia* pneumonia and septicaemia are life-threatening [35, 36]—and in its emergence as a major pathogen in patients with CF [37–39]. By the 1990s, disturbing reports also emerged of fatal cases of *B. cepacia* pneumonia and septicaemia in previously

Table 1. Major issues associated with *B. cepacia* and cystic fibrosis (CF)

- Is there convincing evidence to confirm that *B. cepacia* has pathogenic potential and is not merely a marker of pulmonary disease?
- Based on the success, but unpopularity, of segregation and advances in clarifying the taxonomy of the genus *Burkholderia*, should all *B. cepacia* be treated as equal? Can phenotypic or genomic markers be found which would identify highly transmissible or virulent clones?
- To what degree do natural environments represent a reservoir for *B. cepacia* and a hazard for CF patients? What hazards are associated with the development and use of *B. cepacia* as a biological control agent?
- Could an improved understanding of the host immune response, including enhanced cytokine induction by bacterial surface components, clarify the immunopathology of *B. cepacia* and lead to innovative forms of immunotherapy?
- At present, it is not possible to forecast the clinical outcome of *B. cepacia* colonisation. Can host and bacterial factors responsible for initial colonisation and poor clinical outcome be identified?
- Recently, it has been demonstrated that CF airway epithelia contain bactericidal activity that is inhibited reversibly by high NaCl concentrations. Does this killing potential include *B. cepacia* and is it host or strain specific?
- Ultimately, the identification of bacterial and host factors associated with transmission and virulence would assist greatly in the rational design of an effective *B. cepacia* vaccine.

healthy individuals [40, 41]. Community-acquired *B. cepacia* infections are uncommon, but the organism's pathogenic potential and the financial implications of antimicrobial therapy were recently strikingly demonstrated when an offshore oil worker developed multiple brain abscesses secondary to suppurative otitis media. Therapy involved four neurosurgical operations, an extensive period of hospitalisation and an antibiotic bill of £10K [42].

The above case also demonstrated an interesting and unexplained variability in antibiotic susceptibility profiles that has been observed in serial *B. cepacia* isolates from single patients and during epidemic outbreaks [43–46]. The mechanism responsible for variable susceptibility is unclear, but may be associated with the observation that migration of insertion sequences within the *B. cepacia* genome can affect the expression of genes that modulate antibiotic resistance [47].

B. cepacia and cystic fibrosis

During the last decade, the major clinical interest in *B. cepacia* has focused on its addition to the relatively narrow spectrum of microbial pathogens responsible for debilitating and ultimately fatal pulmonary infections in patients with CF [26, 39, 48, 49]. In the late 1980s, surveillance studies in the UK indicated a maximum prevalence of 7% [39, 50–52]; however, in some CF centres this later increased to approach the prevalence of 40% described in contemporary North American studies [53]. The three major issues concerning *B. cepacia* can be summarised as follows: 1, the clinical risk of rapid and fatal pulmonary decline, even in patients with previously mild disease; 2, patient-to-patient spread of epidemic strains within and between regional CF centres and between centres in the UK and North America; and 3, the innate multiresistance of most *B. cepacia* isolates to available antibiotics—which deprives patients of effective antimicrobial therapy [46, 54]—combined with the failure to reduce the bacterial population in sputum and a relatively poor clinical response even when the colonising strain exhibits in-vitro susceptibility.

The clinical significance of *B. cepacia* in CF patients was first described in 1984 in a seminal report by Isles *et al.* [37]. In addition to noting the increased prevalence of *B. cepacia* colonisation in patients attending Toronto clinics, Isles *et al.* described a rapid and unexpected clinical decline, including necrotising pneumonia and bacteraemia, that occurred in c. 20% of colonised patients. This acute clinical decline is sometimes referred to as 'cepacia syndrome' [37]. It is important to note that acute clinical deterioration and bacterial spread to sites other than the lung is not observed with the other major CF pathogens, *Staphylococcus aureus*, *Haemophilus influenzae* and *P. aeruginosa*.

The second major issue relating to *B. cepacia* arose in the mid 1980s as an increasing—but scientifically unproven—conviction held by some CF carers that the clustering of cases in some large North American clinics had arisen from cross-infection. At that time, an alternative explanation for clustering was the difficulty in culturing this relatively new pathogen from CF sputa [48]. As evidence, in a controlled study involving 115 North American CF centres, only 36 (31%) cultured the organism successfully from a seeded sputum specimen [55]. However, by the early 1990s, the availability of selective culture media [48] and awareness of the organism's cultural idiosyncrasies [56] indicated that regional variation in the prevalence of *B. cepacia* colonisation could not be explained simply by laboratory methodology. Furthermore, the development and use of bacterial fingerprinting techniques—including multilocus enzyme electrophoresis (MLEE), pyrolysis mass spectroscopy, PCR-ribotyping and pulsed-field gel electrophoresis (PFGE)—provided compelling evidence for person-to-person spread of *B. cepacia* through nosocomial and social contacts (Table 2) [25, 37, 46, 57–75] and, occasionally, in the absence of proven sputum colonisation [67]. Epidemiological data also provided scientific justification for the introduction of guidelines by national CF organisations to improve personal and hospital hygiene and, more controversially, for the implementation of segregation policies to limit contact between colonised and non-colonised individuals [76]. Surveillance studies show that segregation undoubtedly reduces the incidence of *B. cepacia* cross-infection [38, 62, 71, 77], but the strategy has not eliminated acquisition. Furthermore, the logistic and social consequences of draconian infection control measures reminiscent of mediaeval approaches to leprosy have not been accepted universally. In particular, the need for such measures has been questioned fiercely by patients and care-givers in CF centres where intensive surveillance has not revealed a high incidence or prevalence of *B. cepacia* colonisation.

A pathogen or a marker of lung disease?

In the 1970s, some microbiologists and clinicians considered *S. aureus* to be the only true microbial pathogen in CF patients and viewed *P. aeruginosa* as merely a marker of disease. A similar doubt has accompanied the emergence of *B. cepacia* and has exacerbated the controversy surrounding segregation of colonised individuals. In discussions of any potential opportunist pathogen, it is easy to find evidence of asymptomatic carriage; even *Salmonella typhi* and *Vibrio cholerae* do not invariably exhibit pathogenicity!

Clarification of the clinical relevance of *B. cepacia* is also thwarted by the fact that the available scientific evidence requires particularly careful analysis. There is an inclination to link bacterial transmissibility and virulence, and to categorise individual *B. cepacia*

Table 2. Evidence for and against person-to-person transmission of *B. cepacia*

Reference	Comments
A. Evidence in favour of person-to-person transmission	
Isles <i>et al.</i> [37]	Seminal paper: noted rising incidence of <i>B. cepacia</i> and cepacia syndrome in Canadian clinics
Thomassen <i>et al.</i> [57]	Fall in incidence after segregation
LiPuma <i>et al.</i> [58]	Prevalence of one ribotype in individual clinics
LiPuma <i>et al.</i> [59]	Ribotyping demonstrates person-to-person spread between two patients at a CF camp
Anderson <i>et al.</i> [60]	Nosocomial outbreak
Millar-Jones <i>et al.</i> [61]	UK nosocomial outbreak
Govan <i>et al.</i> [62]	Genotypic fingerprinting and extensive epidemiological data provides compelling evidence of person-to-person spread through social contact in and between two UK CF centres
Smith <i>et al.</i> [63]	Further UK outbreak with transmission in clinical and social settings
Bingen <i>et al.</i> [64]	International consensus confirming <i>B. cepacia</i> transmissibility
Corkill <i>et al.</i> [65]	Highlights transmission particularly at UK CF events
Pegues <i>et al.</i> [66]	Demonstration of transmission at USA CF camps
Johnson <i>et al.</i> [25]	Intercontinental spread of Edinburgh/Toronto strain ET12
LiPuma <i>et al.</i> [67]	Inapparent transmission from culture-negative patient (?)
Ryley <i>et al.</i> [68]	Further UK outbreak
Sun <i>et al.</i> [69]	Cable pili demonstrated on intercontinental strain (ET12)
Reverts <i>et al.</i> [70]	Prevalent strain in Belgian clinic
Whiteford <i>et al.</i> [71]	Outbreak in UK paediatric clinic
Pitt <i>et al.</i> [46]	Strain ET12 prevalent in UK clinics: accounting for 38% of cases
B. Cases with no evidence of person-to-person transmission	
Glass and Govan [72]	No transmission of pathogenic strain between siblings
Hardy <i>et al.</i> [73]	No transmission to uncolonised patients during hospitalisation
Taylor <i>et al.</i> [74]	No transmission in UK unit before segregation
Steinbach <i>et al.</i> [75]	No transmission in large CF unit despite no segregation of hospitalised patients

strains as either transmissible and virulent, or non-transmissible and avirulent. There is no scientific justification for this view. In epidemic outbreaks in which patients are colonised by the same strain, some patients may remain asymptomatic whilst other individuals succumb to rapid and unexpected fatal deterioration [37, 62]. In the case of transmission, epidemiological evidence has clearly identified lineages with enhanced transmissibility [25, 46, 62, 69]; however, based on present knowledge, it cannot be stated with confidence that a strain inherently lacks the ability for epidemic spread. Furthermore, apparently 'non-transmissible' strains that have not spread even to a patient's CF sibling have been responsible for fatal infection [72]. Finally, it could be argued that transmission is not strain-dependent, but is associated with nosocomial or social opportunities for transmission. This hypothesis is certainly not supported by the behaviour of the particular *B. cepacia* lineage with a notorious ability to spread in CF centres in the UK [46, 62] and North America [25, 69], referred to as the Edinburgh/Toronto lineage [69] or ET12 intercontinental clone (multilocus enzyme electrophoresis type 12) [25]. For convenience, this particular *B. cepacia* lineage will be referred to as the ET12 lineage in the remainder of this review.

Some CF carers who have experienced transmission of *B. cepacia* amongst small numbers of their patients have argued against segregation on the grounds that no significant clinical deterioration was observed and that implementation of such draconian measures stigmatises patients and prevents valuable social contacts with other CF patients [70]. However, the hypothesis that *B. cepacia* is transmissible but merely a marker of pulmonary deterioration can be chal-

lenged. A recent retrospective study of the clinical status of *B. cepacia*-colonised adults in the 24-month period before colonisation found no difference in their lung function, number of days in hospital or outpatient visits [77]. Furthermore, in numerous case-controlled studies involving large numbers of patients, *B. cepacia* colonisation has been associated in some but not all patients with an accelerated decline in pulmonary function and a poor prognosis [71, 77-81]. Most studies have reported that the risk of clinical deterioration on acquisition of *B. cepacia* is increased in adult patients with severe disease [78-80]. This contrasts with an epidemic outbreak of *B. cepacia* among children, in whom the dominant impact on respiratory function was greater in patients with better levels of respiratory function [71]. Explanations for the range of clinical responses associated with *B. cepacia* colonisation and inability to predict the clinical outcome in individual patients could include: 1, differences in strain virulence; 2, the relatively low 20% 'strike rate' of cepacia syndrome; 3, the influence of co-colonisation by other pathogens; 4, the age at which colonisation occurs; 5, individual host immune responses; and 6, the severity of underlying CF disease.

The hypothesis that *B. cepacia* colonisation is merely a marker of severe lung disease is also undermined by the fact that fatalities have occurred in CF adults with mild CF disease, including individuals not already harbouring *P. aeruginosa* [62]. Finally, one of the most striking results from the first microbiological studies in transgenic CF mice showed that 70% of CF mice exposed to *B. cepacia* succumbed to more severe broncho-pulmonary infection than control animals [82].

The Edinburgh/Toronto/ET12 epidemic lineage

In reviewing the emergence of *B. cepacia* in CF populations in Europe and North America, it is necessary to emphasise the influence of epidemic lineages on the incidence and prevalence of *B. cepacia* within CF centres. Evidence shows that the incidence in a centre can be influenced greatly by the epidemic spread of a single lineage, and that if such spread is discounted then the prevalence of *B. cepacia* in most CF centres remains relatively low at 5–10%. Transient colonisation by *B. cepacia* also influences prevalence and occurs in c. 5% of CF patients; however, transient colonisation is observed very rarely with the ET12 lineage (authors' unpublished observations), perhaps reflecting the high colonisation potential of this clone. From a clinical, epidemiological and evolutionary viewpoint, the influence of this single clone on the CF community is considerable. In the UK alone, it has been isolated in eight (50%) of 16 CF centres and from 68 (38%) of 178 *B. cepacia*-colonised patients [46]. Attempts to identify its origins have been frustrated by a lack of stored isolates; however, investigation of available isolates allows several conclusions to be reached. Based on evidence from MLEE and ribotyping [25] and PFGE [46, 62], the first known isolates of this epidemic lineage were cultured from Ontario paediatric patients in the latter half of the 1980s [25]. In the UK, the first recorded isolate of the same lineage was in August 1989 [62] from a patient who had never been out of the UK nor shown any evidence of *B. cepacia* colonisation during previous bacteriological investigations. The patient had previous contacts with other UK patients colonised by *B. cepacia*, but the isolates from these patients were not available.

From the available evidence, it appears that the Edinburgh/Toronto/ET12 lineage was established in Canada before its appearance in the UK, and that at some stage in the late 1980s, intercontinental spread occurred between UK and Canadian patients whilst attending summer camps in Ontario, followed by inter-regional spread in the UK during social contacts at meetings [25, 62]. It is tempting to conclude that this highly transmissible strain is clonally related to the isolates cultured during the first documented outbreak of *B. cepacia* in CF patients in Ontario, reported in 1984 [37].

Potential pathogenic mechanisms of *B. cepacia*

Although *B. cepacia* produces several putative virulence determinants—including haemolysins, proteases, lipases, siderophores and catalase—a major clinical role for these factors has not been demonstrated convincingly in CF [83, 84]. However, catalase is associated with the organism's ability to resist killing by professional phagocytes and to produce serious infection in patients with CGD [85].

Intracellular survival

Several puzzling clinical and scientific observations have led to speculation that *B. cepacia* can survive and grow within pulmonary phagocytes or respiratory epithelial cells. First, clinical resistance to antimicrobial therapy despite demonstration of an isolate's susceptibility *in vitro*; second, isolation of serum-sensitive isolates in bacteremic infection [86]; third, chronic pulmonary colonisation despite a pronounced antibody response [87]; and fourth, the close taxonomic relationship between *B. cepacia* and the intracellular pathogen, *B. pseudomallei*. However, to date, the scientific evidence for intracellular survival or growth of *B. cepacia* is not convincing. Studies of intracellularly in bacterial pathogens can be difficult and, in the case of *B. cepacia*, are complicated further by the organism's innate resistance to antibiotics, including aminoglycosides, which are used commonly in intracellular assays to kill extracellular organisms. As it is known that *B. pseudomallei* survives and multiplies within professional phagocytes [88], studies within our group have focused on monocytes, with *Listeria monocytogenes* and *P. aeruginosa* as positive and negative controls, respectively. However, it was not possible to demonstrate either enhanced uptake or survival of *B. cepacia* in monocytes. Previously, Burns [89] reported the observation of *B. cepacia* within CF post-mortem respiratory epithelial cells by electron microscopy, but no further data have been published to validate this important finding. Low-level invasion *in vitro* of a respiratory epithelial cell line by the epidemic ET12 lineage has been demonstrated [90], but the significance of limited epithelial invasion by bacteria remains unclear [91]. A recent and potentially seminal publication has even suggested that enhanced uptake of CF pathogens by epithelial cells expressing surface cystic fibrosis transmembrane conductance regulator (CFTR), followed by epithelial desquamation, may be an important host defence mechanism rather than a bacterial virulence determinant [92].

Overall, the role of intracellularly in the pathogenesis of *B. cepacia* infection in CF patients is still in doubt. As a caveat, the demonstration of its intracellular survival and growth within amoebae raises the possibility that these free-living protozoa may act as an environmental reservoir from which CF patients could acquire the organism [93].

B. cepacia and host immune responses

Colonisation with *B. cepacia* is associated with a pronounced and specific humoral response, including raised serum IgG and IgA and sputum IgA titres against *B. cepacia* lipopolysaccharide (LPS) and outer-membrane protein (OMP) components [87, 94]. Anti-*B. cepacia* antibodies have also been detected in non-colonised CF patients, and particularly in patients colonised with *P. aeruginosa* [87, 95]. Studies with pre-absorbed sera have failed to demonstrate an appreciable

degree of cross-reactivity between the two species, either for OMP or LPS components [87, 96], suggesting that the response to *P. aeruginosa* is not the source of pre-colonisation anti-*B. cepacia* antibody. Generally, levels of anti-*B. cepacia* immunoglobulin in non-colonised patients are low, but the demonstration of substantially raised titres in a subset of patients may reflect previous exposure to *B. cepacia* where an appropriate antibody response has prevented the occurrence of colonisation. On the other hand, the demonstration of antibody in stored pre-colonisation sera from patients who subsequently became colonised, indicates that antibody does not always play a preventative role. Similarly, the role of antibody in patients once they are colonised is unclear; for example, clinical outcome is independent of the magnitude of anti-*B. cepacia* responses [87]. A recent study [97] with immunoblotting techniques has suggested that IgG antibodies against a 30-kDa OMP, identified presumptively as the major immunodominant porin, OMP D [95, 98], are associated with a better prognosis in colonised patients. If these results are confirmed, it raises the possibility of using this OMP as a target for immunotherapy.

The association of *B. cepacia* with CGD, an inherited defect in neutrophil oxidative killing pathways, and the role of neutrophils as the predominant immune effector cell in the CF lung [99], have led to speculation that the interaction between *B. cepacia* and neutrophils may be important in the evasion of host defences by this organism. Speert *et al.* [85] demonstrated that, unlike *P. aeruginosa*, *B. cepacia* is resistant to non-oxidative neutrophil killing mechanisms; hence the role of *B. cepacia* in CGD. Evasion of the normal neutrophil oxidative burst would aid the survival of *B. cepacia* in the presence of a pronounced immune response. Within the CF lung, normal opsonisation processes are compromised severely through the disruption of immune effector molecules by bacterial and host proteases [100, 101]. In particular, cleavage of complement receptors and immunoglobulin molecules within the respiratory tract may neutralise the humoral immune response to *B. cepacia* and enable the organism to persist in the lungs of colonised patients. However, this observation does not explain the ability of rough, LPS-deficient, serum-sensitive *B. cepacia* to cause invasive pneumonitis and septicaemia in patients with elevated anti-*B. cepacia* immunoglobulin titres [86].

Inflammatory damage

Increasing evidence has emerged to suggest that host immune responses are important in the pathogenesis of *B. cepacia* infection. A UK multicentre study has shown that levels of the inflammatory markers, C-reactive protein and neutrophil elastase $\alpha 1$ -antiproteinase complex, are significantly higher during *B. cepacia*-associated exacerbations than in exacerbations

caused by *P. aeruginosa* alone. Aggressive antibiotic treatment with the most active agents available did not eliminate *B. cepacia*, but in most cases was associated with a decline in inflammatory markers to pre-exacerbation levels [102]. In addition, anecdotal evidence indicates that patients who exhibit rapid pulmonary decline and pronounced inflammatory symptoms, but who do not respond to antibiotic therapy, nevertheless respond to treatment with commercial preparations of immunoglobulin. The relative absence of *B. cepacia* antibodies in healthy human donors [87], from whom these immunoglobulins are obtained, suggests that such preparations contain potentially useful anti-inflammatory activity.

An unexpected but informative result from our own studies has demonstrated that LPS from clinical and environmental isolates of *B. cepacia* induces pro-inflammatory cytokines, including the major cytokine tumour necrosis factor α (TNF α), to a level 10-fold that induced by *P. aeruginosa* LPS and matching the inflammatory power of *Escherichia coli* endotoxin [103, 104]. The mechanism involved in *B. cepacia* cytokine stimulation is unclear, but is independent of CD14 receptors. Of interest, induction of TNF α by *B. cepacia* LPS is reduced in the presence of *P. aeruginosa* LPS, suggesting that the diversity of clinical outcomes in patients colonised with *B. cepacia* may be influenced in part by the presence or absence of *P. aeruginosa* and other CF pathogens [105].

What is a true B. cepacia?

Further research to establish a gold standard for laboratory identification of *B. cepacia* has assumed high priority. Reliable identification is important not only in attempts to clarify the organism's pathogenic potential, but also because of the clinical, social, psychological and potentially litigious consequences for patients, carers and diagnostic laboratories associated with the organism's acquisition and transmission. Selective media and laboratory protocols for culture and presumptive identification of *B. cepacia* from clinical or environmental sources have been described and their value in microbiological surveillance established [14, 48, 56, 106]. However, existing selective media also support the growth of other gram-negative non-fermenting bacilli [46, 48, 56] and unequivocal identification of *B. cepacia* by multitest commercial systems can present difficulties [44, 56, 106, 107].

There is increasing evidence that organisms presently identified as *B. cepacia* by standard laboratory procedures exhibit such diverse genotypic and phenotypic properties that attempts to generalise on virulence, transmission and antibiotic susceptibility are ill-founded. Simpson *et al.* [44] speculated that epidemic strains may represent a *B. cepacia* sub-

population, arising as bacterial hybrids or through horizontal transfer of virulence genes from the closely related pseudomonads *B. gladioli* and the highly dangerous intracellular pathogen *B. pseudomallei*. Recently, isolates identified as *B. cepacia* were characterised further by analysis of cellular proteins and fatty acid components and clustered by means of computer-assisted numerical comparison of the profiles. Representative isolates from individual clusters were selected to determine genotypic relatedness within and between clusters by means of DNA-DNA and DNA-rRNA hybridisation assays. These molecular phylogenetic studies revealed that organisms identified by conventional tests as *B. cepacia* comprised several new *Burkholderia* spp. [108].

According to taxonomic conventions, new species names should not be given to bacteria that cannot be identified reliably by phenotypic characteristics; instead, such groups can be described by the terms genomovar I, II, etc. [109]. Following this convention, isolates identified as *B. cepacia* by conventional multitest systems such as the API 20NE system (API-bioMérieux, Marcy l'Etoile, France) constitute at least four different genomovars of *B. cepacia*; other presumed *B. cepacia* strains are identified as the nitrogen-fixing organism *B. vietnamiensis*. Preliminary studies on a small number of isolates have indicated that the majority of CF isolates from Belgium and the UK tend to cluster in genomovar III [70, 108]. Subsequent ongoing analyses of a larger collection of environmental, phytopathogenic and clinical isolates in our laboratories have confirmed the potential importance of genomovar identification. For example, the isolate responsible for the first UK report of *cepacia* syndrome [72], and three individual epidemic clones including the highly transmissible ET12 lineage [25, 44, 62, 69] each belong to genomovar III. It should be stressed that *B. vietnamiensis* and the remaining *B. cepacia* genomovars were also identified amongst isolates from CF patients, and that genomovar III status is not synonymous with high transmissibility [72]. Of the 150 '*B. cepacia*' isolates studied to date, most environmental isolates (including the phytopathogenic type strain ATCC 25416) belong to genomovar I; in contrast, isolates associated with acute clinical decline in CF patients are restricted to genomovar III. These results confirm the complex taxonomic heterogeneity within the genus *Burkholderia* and have important diagnostic implications for infection control in the CF community.

Unique bacterial clones and *B. cepacia* transmission factors

Epidemiological data and genomic fingerprinting suggest that the variable incidence of *B. cepacia*—in particular, the lack of cross-infection in some centres

[75, 81], and the contrasting epidemic spread in others—reflects the behaviour of a relatively small number of highly transmissible clones [46, 69, 110–112].

It seems reasonable to speculate that *B. cepacia* strains responsible for epidemic spread may harbour a common colonising factor whose identification could be exploited for diagnostic and therapeutic purposes. At present, the most significant of these factors is adhesion to respiratory mucin [53, 113–115], associated with giant intertwined fibres referred to as cable pili [53, 114]. The gene responsible for cable pili, *chl*, has been detected in the highly transmissible ET12 lineage, represented by the Edinburgh isolate CF5610 (J2315) [16, 25, 62, 69, 115], and responsible for *B. cepacia* colonisation in 38% of UK patients [46]. In a slightly different form, *chl* has also been associated with epidemic transfer of *B. cepacia* from CF to non-CF patients in a Mississippi outbreak [16, 69, 115]. However, studies with a *chl* DNA probe indicated that *chl* is not present in all epidemic clones, suggesting that other bacterial and host factors need to be identified [69]. Interestingly, a recent study [116] has described enhanced binding of the ET12 lineage to lipid receptors, particularly the galactolipid globotriolysylceramide (GB₃), and led to speculation that upregulation of GB₃, mediated through the infection process and TNF stimulation within the lung, may provide an alternative receptor for isolates in which cable pili are poorly expressed and a second receptor system for the epithelial attachment of bacteria that have migrated through the mucosal blanket.

Experimental proof of direct or indirect transmission of epidemic *B. cepacia* is not feasible and can be judged only by circumstantial evidence. However, epidemiological data has strikingly demonstrated such potential. Colonisation with more than one strain of *B. cepacia* is unusual and has been reported in <10% of patients [46]. During the Edinburgh outbreak, PFGE fingerprinting showed that one patient harboured two *B. cepacia* strains in his respiratory secretions, including the ET12 clone; however, only the epidemic strain was transmitted subsequently to his girlfriend [62].

Modes of transmission and the risks of acquisition

The potential risks of *B. cepacia* transmission, either directly by person-to-person spread or indirectly from contaminated fomites, continue to be a major concern to the CF community. Table 2 summarises the extensive documented evidence for direct transmission of *B. cepacia* between CF patients during close contacts within hospitals [61, 63, 65], at educational or summer camps [59, 66] and through other social contacts [62, 63]; in contrast, several reliable studies have found no evidence of cross-infection [72–75]. In

their initial report, LiPuma *et al.* [59] cited previous failures to culture *B. cepacia* from respiratory equipment and environmental surfaces as circumstantial evidence that direct person-to-person spread might be the primary means of transmission. However, a subsequent prospective study [117] with selective culture and DNA-based typing of isolates showed that colonised patients can contaminate their environment; thus indirect transmission might occur via contaminated surfaces. The intrinsic resistance of *B. cepacia* to many antibiotics also raised justifiable concern that the use of contaminated home-use nebulisers might present a special hazard for *B. cepacia* acquisition. Currently, evidence for nebuliser-associated transmission is scanty and equivocal. A case-controlled retrospective study of five CF patients undergoing treatment in a CF centre [118] showed a significant association between outpatient nebuliser use and *B. cepacia* colonisation. *B. cepacia* was also cultured from nebulisers used by colonised patients. Unfortunately, no bacterial typing was performed to confirm the clonal relationships of the human and nebuliser isolates. Recently, in a prospective study [119], *B. cepacia* was cultured from three of 35 home-use nebulisers. DNA macrorestriction analysis by PFGE revealed that one of two strains of *B. cepacia* recovered from the nebuliser of one patient was also present in the patient's sputum. However, sputum cultures from the two other patients whose nebulisers harboured *B. cepacia* did not yield the organism, suggesting an environmental origin for the *B. cepacia* strain isolated from the nebuliser. Other studies of nosocomial acquisition of *B. cepacia* in non-CF patients have suggested that respiratory infection probably occurred by indirect transmission following use of contaminated nebuliser devices [31, 120]. Airborne dissemination may also present a small risk of *B. cepacia* acquisition. In a prospective study, *B. cepacia* was recovered from the room air during occupation by five of six patients, but to only a limited extent, with the number of bacteria ranging from 1 to 158 cfu/m³ [121]. Maximum yields were associated with episodes of coughing and, after a patient left the room, the organism persisted in room air for up to 45 min.

To conclude, ethical considerations prevent experi-

ments that could provide scientific data to assess the risks of *B. cepacia* acquisition, including clarification of the frequency of contact and the infectious dose required. Based on accumulated evidence (Tables 2 and 3), skin contact, respiratory aerosols, sharing food, contaminated equipment, co-habitation or undergoing physiotherapy in the same room as a *B. cepacia*-positive individual present reasonable risks of acquisition. However, epidemiological evidence [38, 62], including the high numbers (typically >10⁸ cfu/ml) of *B. cepacia* present in the saliva of colonised patients, suggests that the close and frequent social contact that occurs between siblings, the direct exchange of respiratory secretions associated with kissing, and the involvement of a highly transmissible *B. cepacia* lineage arguably present the greatest risks of acquisition.

Environmental release of *B. cepacia* as a biological control agent

Whilst the CF community debates the clinical issues of *B. cepacia* colonisation and transmission, agricultural microbiologists continue to develop the organism as a biological control agent to exploit its antifungal activity (Fig. 1) for the enhancement of crop yields [122, 123] and its nutritional adaptability in the bioremediation of landfill sites, contaminated soils and ground water aquifers [124–126]. Deliberate environmental distribution of *B. cepacia* as field inoculants raises the issue of the phylogenetic relationship between *B. cepacia* of environmental and clinical origin and the potential hazard for human infection. The debate on this relationship has revealed the gulf that exists between different areas of interest and microbiological expertise and, as stated recently in an editorial comment on another contentious issue, bovine spongiform encephalopathy, 'underscores the weakness of separating agricultural and medical science' [127].

We have stated previously that the scientific evidence that environmental strains of *B. cepacia* present little hazard to man is weak [14] and is based on examination of only a few bacterial isolates and

Table 3. Factors that may influence acquisition of *B. cepacia*

- In colonised individuals, *B. cepacia* saliva counts can exceed 10⁸ cfu/ml, suggesting that the highest risk of patient-to-patient spread is transmission of respiratory secretions during kissing or through sharing of eating or drinking utensils.
- Spirometer mouthpieces become heavily contaminated during lung function tests. Risk avoided by use of disposable mouthpieces. Recovery from the surface of lung function equipment is low.
- Recovery from antibiotic reservoirs of nebulisers has been reported, but incidence is low and the degree of risk appears secondary to the preceding factors.
- Aerosol recovery is low, suggesting low risk of aerosol transmission.
- Hands become contaminated after coughing and the organism can be transmitted by handshake. Survival on hands reduced to 10% after 30 min; this varies in different individuals and may account for variable recovery in surveillance studies.
- Gastrointestinal carriage has not been demonstrated, even in colonised individuals, suggesting that the risk of faecal-oral spread is minimal.
- After surface contamination with *B. cepacia*-containing sputum, viable bacteria can be recovered for several weeks.
- Surface contamination by *B. cepacia* sputum is eliminated by treatment with UV irradiation and with common hospital disinfectants, including Milton, Dettol, alcohol 70%, phenols, iodine and cetrimide. Careful drying is important after washing or disinfection.
- Recovery of *B. cepacia* from soil, plants, drains, lakes and surface waters is low, suggesting that natural environments present a possible but low risk for acquisition.

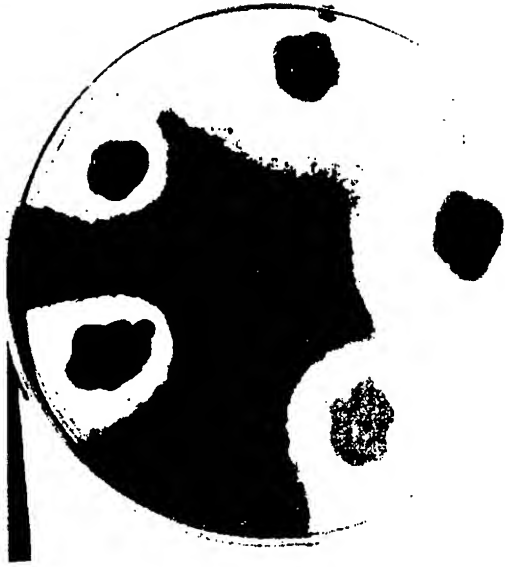


Fig. 1. Inhibition of the phytopathogenic fungus *Rhizoctonia solani* by five isolates of *B. cepacia*. The fungus was inoculated in the centre of the plate and bacteria around the perimeter. Cultures were photographed after incubation for 14 days at room temperature.

inappropriate bacterial properties [24]. Although some studies have indicated that environmental and clinical isolates are distinct, no reliable phenotypic markers have been identified [25, 45, 108]. The suggestion that clinical isolates can be distinguished from soil isolates based on the former's lack of plant pathogenicity [45] is discounted by the fact that CF isolates of *B. cepacia* will readily macerate onion tissue (Fig. 2) [14]. In addition, the invasive *B. cepacia* foot lesions

known as swamp foot [128], acquired by military personnel during jungle training, confirm the pathogenic potential of environmental strains of *B. cepacia* for man.

The potential hazard that some or all environmental *B. cepacia* strains present to the CF community is unclear and requires investigation. The fact that new cases of *B. cepacia* colonisation continue to occur with strains that show no genotypic relationship to other isolates within the same CF centre, points to the environment as a potential source. However, the extent of this risk is difficult to assess. Extensive microbiological safaris into supermarkets and domestic homes [15], and a range of botanical soils and cultivars [14], indicate that *B. cepacia* can be cultured from up to 20% of warm moist environmental sites, particularly soils, but that it is not as ubiquitous as other pseudomonads. Interestingly, in our studies to date, none of the environmental isolates of *B. cepacia* have been identified as belonging to genomovar III.

Conclusions and future prospects

B. cepacia is a striking example of a multiresistant soil saprophyte and phytopathogen that has emerged as an important threat to susceptible human hosts. In the CF community, the degree to which infection control measures should be implemented continues to arouse strong scientific and social debate. The validity of strict control is supported by circumstantial, but nevertheless compelling, evidence for direct person-to-person transmission of epidemic strains through nosocomial and social contact. In contrast, although the risk of indirect iatrogenic spread from contaminated fomites remains

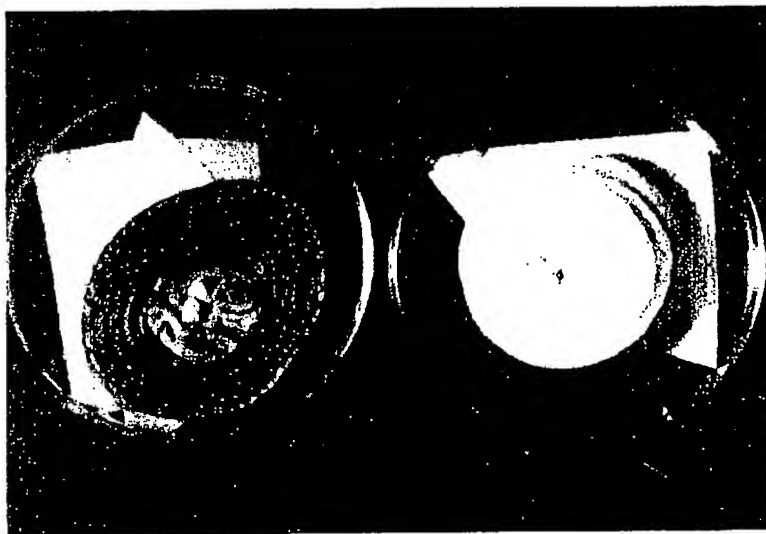


Fig. 2. Soft rot of a segmented 'compromised' onion inoculated with a clinical *B. cepacia* isolate of the epidemic ET12 lineage (left) and an uninoculated control (right), both incubated at 30°C for 72 h. Reproduced with permission from Butler *et al.* [14].

unclear, available evidence suggests that this route is less important than direct transfer. An important caveat in attempts to generalise on *B. cepacia* transmission is evidence that the major epidemics of *B. cepacia* involve a subpopulation of highly epidemic lineages which might be re-allocated ultimately to new species; '*Burkholderia cfe*' might be an appropriate but probably controversial choice! Ongoing microbiological surveillance in CF centres indicates that sporadic acquisition of epidemic lineages continues to occur when there is a failure to comply with infection control measures. For example, a striking demonstration of the continued potential for transmission of the ET12 lineage was its recent acquisition by an Edinburgh CF adult; extensive inquiries suggested that this patient had social contact for only 10 min whilst visiting another CF male who was hospitalised during an episode of *B. cepacia* septicaemia. Even when infection control appears effective in preventing spread of epidemic lineages, new cases of *B. cepacia* colonisation continue to occur with isolates that exhibit unique PCR ribotyping or PFGE profiles. Such sporadic acquisitions raise a fundamental question concerning the source and colonising potential of individual *B. cepacia* strains. For example, does the environment contain a subpopulation of *B. cepacia* clones that are innately primed for human colonisation, or does colonisation and virulence in man require in-vivo adaptation? Future improvements in laboratory identification of *B. cepacia* subpopulations associated with CF disease and identification of transmission factors, in addition to cable pili, may provide scientific justification for relaxation of segregation in the absence of known epidemic and potentially virulent lineages. Turning our attention to CF patients, we need to clarify why colonisation by the same strain of *B. cepacia* leads to variable clinical responses, ranging from asymptomatic colonisation to rapid fatal pulmonary deterioration. It could be argued that this particular problem is not unique to *B. cepacia*, and that applying Koch's postulates in an attempt to distinguish between sycophancy and pathogenic potential is difficult when dealing with any opportunist pathogen. Certainly, host factors cannot be ignored in attempts to understand the pathogenic processes involved in CF lung infections.

During the final preparation of this review, a deceptively simple and elegant study has illustrated how CFTR-associated defective Cl^- transport across airway epithelia might lead to bacterial colonisation in CF patients. Smith *et al.* [129] showed that the normal human apical epithelial surface is bactericidal for *P. aeruginosa* and *S. aureus*; in contrast, the bactericidal activity was inhibited reversibly in CF epithelia because of a high NaCl concentration. If this phenomenon varies in individual CF patients—or if individual *B. cepacia* strains differ in susceptibility to the defensin-like bactericidal agent—it might explain some of the host- and pathogen-specific anomalies associated with *B. cepacia* pulmonary infection and

suggest novel strategies for infection control and therapy of this unusual and challenging opportunist pathogen.

It is difficult to avoid a final comment on the irony that whilst *B. cepacia* continues to hold the CF community to ransom, agricultural microbiologists seek to develop the commercial and beneficial potential of this microbial Jekyll and Hyde in their search for biological control agents. This situation demonstrates the diversity of microbiology, but should also encourage attempts to reduce the present gulf between agricultural and medical science.

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Note added in proof

Following submission of this review, the results of an epidemiological study of *B. cepacia* in a large series of CF patients attending the CF centre in Verona were published. Cazzola *et al.* concluded that their results are difficult to interpret. Nevertheless, data are essential if progress is to be made in unravelling *B. cepacia* epidemiology, and the results of this study are particularly relevant to the major issues discussed in our review.

Between Nov. 1991 and Dec. 1994, *B. cepacia* was cultured from 85 (11.0%) of 769 CF patients attending the Verona centre. Based on genomic fingerprinting, 32 (53.3%) patients were colonised by individual *B. cepacia* strains; the remaining 28 (46.7%) patients were divided into 10 subgroups, each colonised by a distinct strain. As previously encountered with the ET12 lineage, the outcome of *B. cepacia* colonisation in the Verona study varied from rapidly fatal septicemia to maintenance of reasonably stable respiratory function, even in patients colonised by the same strain. Cazzola *et al.* provide further evidence for hypotheses discussed in our review that some *B. cepacia* strains exhibit and low transmissibility that the environment is a likely source of sporadic new cases: e.g., transmission was observed in only three of eight pairs of CF siblings; in unrelated patients, direct person-to-person transmission was evident in only 10 cases (16.7%); despite a strict segregation policy, whether as in- or out-patients, 15 new colonised patients were identified during 1993. Considering social implications and the paucity of previous data, it was particularly interesting to note that transmission was demonstrated between two unrelated CF schoolmates.

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Pseudomonas cepacia: Biology, mechanisms of virulence, epidemiology

Pseudomonas cepacia, originally described as a plant pathogen, has emerged as an important cause of infection in altered hosts, particularly in the hospital setting. This organism's ability to survive and proliferate in a variety of solutions, medications, and even disinfectants and antiseptics has resulted in numerous clusters of common-source nosocomial infections. Many patients exposed to *P. cepacia* are merely colonized, but serious infections, including surgical and burn wound infections, bacteremia, meningitis, pneumonia, peritonitis, and urinary tract infections, are not rare. The virulence properties of this pathogen remain poorly characterized. Recently, *P. cepacia* has been reported in some cystic fibrosis centers as an increasingly frequent pulmonary pathogen. This trend has caused considerable concern because of reports of occasional cases of fulminant necrotizing pneumonia and bacteremia. Conversely, many patients with CF who become colonized with this organism have no ill effects. The epidemiology of *P. cepacia* in the CF population is unclear, but some patients probably acquire the organism from colonized siblings with CF. Circumstantial evidence suggests that the organism may also be acquired in the hospital. Treatment of infections is exceedingly difficult, particularly in patients with CF, because *P. cepacia* is resistant to a broad range of antibiotics. (J PEDIATR 1986;108(2):806-812)

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Pseudomonas cepacia, first described as a cause of soft rot in onions in 1950,¹ is now recognized as an important opportunistic pathogen in hospitalized patients and other compromised hosts. Although many patients are colonized with *P. cepacia* without noticeable ill effects, others experience severe, life-threatening infections. Recent reports from a number of centers²⁻⁷ indicate that patients with cystic fibrosis are at particularly high risk for colonization and infection with *P. cepacia*. Indeed, the CF population is the principal group affected by *P. cepacia* at the present time. The difficulty in treating *P. cepacia* pulmonary infection in these patients has stimulated increasing interest in the microbiology, epidemiology, and virulence of this organism.

P. cepacia has had a variety of names in the past, including *Pseudomonas kingii*, *Pseudomonas multivorans*, and EO-1 (eugonic oxidizers group 1), but the identity of all of these organisms has been clearly established.^{8,9} On the basis of nucleic acid homologies, *P. cepacia* has been found to be closely related to *Pseudomonas mallei* and *P. pseudomallei* (the agents of glanders and melioidosis, respectively), *P. pickettii* (a rare cause of

CF Cystic fibrosis

human infections), and several pseudomonal plant pathogens.^{10,11} It is only distantly related to the much more common and well-known opportunist, *Pseudomonas aeruginosa*. Like other members of its genus, *P. cepacia* is ubiquitous in the environment, where it is frequently associated with soil, water, and plants. It is an incredibly versatile organism, capable of utilizing a wide variety of nutrients for growth. Suitable substrates range from sim-

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ple salts such as ammonium acetate¹² to complex organic molecules such as 2,4,5-trichlorophenoxyacetic acid, the principal herbicide in "agent orange."¹³ Even penicillin can serve as the sole source of carbon, nitrogen, and energy.¹⁴ This metabolic versatility has been exploited by genetic engineering of *P. cepacia* strains that utilize environmental chemical contaminants.

P. cepacia survives in a variety of adverse conditions (Table I). It thrives in natural water sources^{15,16} and can proliferate in tap or distilled water,^{17,18} presumably by utilizing trace elements and low concentrations of organic materials. This characteristic, coupled with its resistance to a variety of commonly used disinfectants, antiseptics, and preservatives, makes *P. cepacia* a formidable nosocomial pathogen.

Numerous outbreaks of *P. cepacia* colonization and infection have been caused by contaminated solutions, pharmaceuticals, and medical devices.¹⁹ Contaminated dilute aqueous quaternary ammonium disinfectant has caused epidemics of infection, ultimately leading the Centers for Disease Control to recommend against its use in hospitals.^{18,20} In one instance, *P. cepacia* persisted for 14 years in an inorganic salts solution "preserved" with 0.05% benzalkonium chloride.¹² By gradually increasing the concentration of benzalkonium chloride, strains were easily derived that grew in 16% disinfectant. *P. cepacia* can also persist in aqueous chlorhexidine^{21,22} and povidone iodine.²³

P. cepacia is virtually nonpathogenic in normal, healthy individuals. Even direct injection of contaminated fluids into immunocompetent patients may result in only transient fever or colonization.^{19,22} However, when patients have severely altered host defenses (as in the case of burn victims or patients with CF) or have indwelling catheters or other medical devices, serious infections may occur. Reported infections include endocarditis (particularly in intravenous drug abusers),²⁴ bacteremia,²⁵ postoperative and burn wound infections,²⁶ peritonitis,²⁷ osteomyelitis and septic arthritis,²⁸ meningitis,²⁹ and lung abscess and pneumonia.³⁰⁻³² The infections complicating chronic granulomatous disease³³⁻³⁵ are particularly interesting because they tend to occur in individuals who have had no prior evidence of a host defense problem.

The association of *P. cepacia* with pulmonary disease in CF was first noted during a study of aminoglycoside resistance in pseudomonads isolated from sputum cultures in this population.² Of the 200 patients studied from 1974 to 1978, eight were found to be colonized with *P. cepacia*. More recently, some cystic fibrosis centers have reported a dramatic increase in the rate of isolation of *P. cepacia* from sputum. For example, at the Rainbow Babies and Childrens Hospital in Cleveland the annual incidence of

Table I. Sources of isolates of *Pseudomonas cepacia* (selected)

Decayed onions	Ultrasonic nebulizers
Soil	Infant incubators
Rotting tree trunks	Respirators
Tap water	Humidifiers
Distilled water	Pressure transducers
Saline solution	Intravenous fluids
Raw and pasteurized milk	Normal serum albumin
Topical anesthetics	Cryoprecipitate
Aerosol antibiotics	Dialysis machines
Methylprednisolone	Disinfectants
Urinary catheter kits	Baby lotions

Adapted from Gilardi GL. Lab Management 1983;21:29-32.

pulmonary colonization with *P. cepacia* increased from 3.3% to 8.2% from 1979 to 1983, and the prevalence of patients with *P. cepacia* in their sputum increased from 5.1% to 20.0%.³⁶ An increase in the prevalence of *P. cepacia* colonization also was reported by the CF group at the Toronto Hospital for Sick Children during a similar period.^{5,6} Despite these reports, the magnitude of the *P. cepacia* problem in CF remains undefined. It is possible that the apparent increase in the prevalence of *P. cepacia* in some centers is, at least in part, an artifact of increased laboratory awareness, and this may be exacerbated now that efficient selective culture media are available.^{37,38} Most centers have noted only sporadic isolates of *P. cepacia*. In other centers, such as St. Christopher's Hospital for Children in Philadelphia, the incidence of *P. cepacia* isolations has not changed dramatically, and the prevalence of colonization has gradually declined (Dr. D. Schidlow, personal communication).

The appearance of *P. cepacia* in the CF population has caused considerable alarm, not only because of the increasing number of colonized patients being recognized at some centers but also because some investigators have reported that colonization tends to be associated with accelerated clinical deterioration. Thomassen et al.,³⁹ in Cleveland, noted that patients colonized with *P. cepacia* seemed to have more serious lung disease and a poorer prognosis than patients colonized only with *P. aeruginosa*. Isles et al.,⁶ in Toronto, found that some patients, particularly girls and young women, who acquired *P. cepacia* experienced a rapid decline in pulmonary function.⁶ A small subset of patients who had been clinically stable with relatively mild lung disease experienced an acute, fulminant course characterized by high fever, elevated sedimentation rate and white blood cell count, progressive pulmonary failure, and death. Pathologic examination of the lung revealed severe necrotizing pneumonia. From 1977 to 1981, isolation of *P. cepacia* from patients who died increased from 17% to 42%. Similarly, four young adult women in Cleveland had

relentless deterioration, leukocytosis, spiking fevers, and death within several weeks to a few months after colonization with *P. cepacia*.⁴⁰ *P. cepacia* was recovered from blood cultures in all four patients. *P. cepacia* bacteremia in CF patients is striking; *P. aeruginosa*, the organism that colonizes the vast majority of patients with CF, virtually never causes bacteremia.^{3,41}

Although these reports are of great concern, many patients colonized with *P. cepacia* have no detectable change in their clinical course,^{39,42} and certain patients colonized with *P. aeruginosa* experience unpredictable, sudden pulmonary deterioration. Moreover, regardless of whether the lung is colonized with *P. cepacia* or *P. aeruginosa*, CF pulmonary disease is ultimately lethal. Carefully controlled epidemiologic studies in a large CF population will be required to establish the true virulence of *P. cepacia*. A recently completed study at St. Christopher's Hospital for Children, performed in collaboration with the Centers for Disease Control, represents an important first step.⁷ This case-control study suggested that patients colonized with *P. cepacia* tend to be hospitalized longer and die sooner than comparison subjects matched for severity of illness. However, the matching criteria used in this study (mild, moderate, and advanced disease categorized by Schwachman score⁴³) are broad and subjective, and it may be difficult for any single center to define a sufficient number of controls matched by more precise indicators of the severity of underlying pulmonary disease.

It is not clear how patients with CF initially become colonized with *P. cepacia*. Patients probably encounter the organism frequently in the community, and colonization may be favored by the selective pressure of the prolonged oral antibiotic therapy. Accumulating evidence suggests that many patients acquire *P. cepacia* either in the hospital or through contact with colonized siblings. The clustering of *P. cepacia* in a few centers implies that acquisition of the organism may be associated with specific hospital exposures or practices. Moreover, colonization appears to be correlated with recent or concurrent hospitalization^{7,36,39} or a recent visit to a hospital clinic (Dr. D. Schidlow, personal communication). In studies not involving CF patients, contaminated topical anesthetics⁴⁴ and solutions used in bronchoscopy, aerosolized antibiotics,⁴⁵ and contaminated nebulizers^{40,46} all have been associated with *P. cepacia* pulmonary colonization and infection. Inadequate disinfection of inhalation therapy equipment was an important cause of gram-negative pulmonary infection until the danger was recognized in the last decade.⁴⁷ Therefore, investigators in several centers performed extensive culture surveys to detect *P. cepacia* contamination of the

hospital environment. To date there has been no convincing evidence for an environmental reservoir in CF centers.

Attempts to demonstrate person-to-person spread of *P. cepacia* have yielded tantalizing clues, but the extent of transmissibility among CF patients is unclear. The risk of *P. cepacia* colonization is significantly increased if a sibling harbors the organism.^{7,36,39} Analysis by biotyping, serotyping, bacteriocin typing, and antibiograms suggests that some sibling pairs are colonized by the same strain.⁴⁸ Aside from this apparent sibling-to-sibling spread, direct evidence that *P. cepacia* is transmitted from person to person, either in the hospital or in other settings where CF patients congregate, has been difficult to obtain. Nevertheless, the possibility that *P. cepacia* might be acquired by contact with colonized patients has produced considerable anxiety in the CF community. In Cleveland, the CF group has responded by instituting a variety of procedures to reduce the risk of transmission, including physical separation of colonized and noncolonized patients in the hospital, reeducation of the staff concerning basic infection control procedures, and institution of separate summer camps. These measures have been associated with a sharp decline in the incidence of *P. cepacia* colonization, from 8.2% in 1983 to 1.7% in 1984.³⁶ Further studies are needed to confirm the efficacy of these precautions, which significantly alter the care routine and social life of CF patients.

Antimicrobial therapy for *P. cepacia* infections poses a significant challenge, because this pathogen is routinely resistant to many agents. In fact, resistance to the polymyxins and aminoglycoside antibiotics is useful in distinguishing this organism from other pseudomonads in the laboratory and in designing selective culture media.^{37,38,49} *P. cepacia* is also resistant to first- and second-generation cephalosporins and traditional antipseudomonal penicillins, such as ticarcillin. This is not surprising, because an inducible β -lactamase has been demonstrated in *P. cepacia*, which hydrolyzes many cephalosporins and penicillins.⁵⁰ Until recently, the most effective antibiotics available for the treatment of *P. cepacia* infections were trimethoprim-sulfamethoxazole and chloramphenicol^{19,44,51,52}; these antibiotics may still be useful in certain clinical settings. Treatment of CF poses a special problem; few strains remain susceptible to TMP-SMZ or chloramphenicol, and the aminoglycoside and penicillin antibiotics used to treat *P. aeruginosa* infections will increase the selective pressure favoring colonization with *P. cepacia*. Further studies are required to determine how the growing popularity of aerosolized antibiotics, such as polymyxin, carbenicillin, and tobramycin, in CF centers may affect

Table II. Serotyping of *Pseudomonas cepacia*

Isolate source	Serotype* (%)						Multi-type	Non-typable	Reference
	I	Ia	Ib	II	III	IV			
CF patients (n = 112)	50.9	2.7	0.9	0	1.8	3.6	33.8	6.3	Klinger et al. ⁵⁵
Non-CF patients (n = 65)	7.6	6.2	3.1	0	10.8	7.7	33.8	30.8	Klinger et al. ⁵⁵
Environmental (n = 34)	8.8	8.8	0	0	2.9	0	8.8	70.7	Klinger et al. ⁵⁵
Non-CF patients (n = 137)	21.4	31.7	30.3	8.3	8.3	†	‡	‡	Jonsson ⁵⁴

Serotyping scheme of Jonsson⁵⁴; serotype IV described in Klinger et al.⁵⁵

*Percentage of total strains (per source group) for indicated serotype.

†Serotype IV not described at time of study.⁵⁴

‡Polyagglutinating (multitype) and nontypable strains not indicated.⁵⁴

colonization with *P. cepacia*.^{45,53} In addition, aerosol equipment might become contaminated with *P. cepacia*, resulting in aerosolization of the organism into the lung.^{47,54}

Recently developed antibiotics, including several third-generation cephalosporins, thienamycin (imipenem), aztreonam, and ciprofloxacin, demonstrate some in vitro activity against *P. cepacia*.⁵⁵⁻⁵⁹ Of these agents, ceftazidime was believed to be particularly promising because most strains from patients with CF are susceptible in vitro.⁵⁸ Initial clinical experience with ceftazidime in the treatment of patients with *P. cepacia* infection, however, has been variable. Gold et al.,⁶⁰ administered 18 courses of ceftazidime to 14 CF patients with severe chronic lung disease. Clinical improvement occurred with six treatment courses. Eight treatment courses were classified as failures, and four patients died after six, seven, eight, and 29 days of therapy, respectively. These therapeutic failures could not be attributed to the development of resistance to ceftazidime because resistance was found in only one patient.⁶⁰ Subsequently, the same group treated a less severely ill population of colonized patients.⁴² Most patients improved clinically, as generally can be expected when CF patients are hospitalized and given antibiotics intravenously.⁶¹ Ceftazidime significantly decreased *P. aeruginosa* in the sputum, but there was no reduction in the density of *P. cepacia*. Kercsmar et al.⁶² and Blumer et al.⁶² also noted clinical improvement after ceftazidime administration in the majority of patients who had failed to respond to conventional therapy.

Inasmuch as none of the antibiotics currently under development is likely to have a major impact on *P. cepacia* pulmonary infection in patients with CF, future research will focus on ways to prevent acquisition. Standard infection control techniques will eliminate the usual hospital reservoirs of *P. cepacia*. Barrier precautions may prove useful in limiting person-to-person transmission, should this prove to be a frequent occurrence. The development of

stable, reliable microbiologic typing systems would greatly facilitate study of the epidemiology of *P. cepacia* and permit a more rational approach to preventing further spread of the organism in the population with cystic fibrosis. Several typing systems have been proposed, but all must be considered preliminary. The first system to be described was based on 139 human isolates of EO-1 from the collection of the Centers for Disease Control.⁶⁴ Five serotypes (I, Ia, Ib, II, and III) were described, with types Ia and Ib most frequently encountered. Recent studies have defined an additional serotype, IV.⁶⁵ When recent isolates from CF patients, other patients, and environmental sources were serotyped, it was discovered that a large proportion of the CF isolates were type I, compared with only 8% of strains from other sources (Table II). Many strains (particularly from environmental sources) agglutinated in multiple antisera or were nontypable, limiting the usefulness of this system in its present form and suggesting the existence of additional serotypes.

Two other serotyping systems have been described in the past few years and have been used in limited epidemiologic studies. A French group⁶⁶ serotyped 285 isolates using a combination of seven O (somatic) and five H (flagellar) antisera. Strains from nosocomial outbreaks of *P. cepacia* in medical and surgical units in the Strasbourg area tended to have the same serotypes. For example, strains from a cluster of infection caused by contaminated bronchoscopes were serotype O5H5. Another system of 10 serogroups has been described by Japanese investigators.⁶⁷ With this system all but 12% of 105 strains could be serotyped.

Two biotyping systems have been proposed for *P. cepacia*.^{68,69} These systems are appealing because each relies on a small number of biochemical reactions that could be performed easily by most laboratories. With these systems *P. cepacia* can be separated into four^{68,69} and eight⁶⁹ biovars, respectively. *P. cepacia* can also be charac-

terized by bacteriocin production and sensitivity.⁷⁰ Very recently, Govan et al.⁷¹ reported that 95% of 400 strains were typable, falling into 44 cepacia types. Strains from documented outbreaks tended to have the same bacteriocin production and sensitivity patterns.

Very little is known about the virulence properties of *P. cepacia*. In the initial report by Burkholder¹ and in several subsequent studies,⁷² 50% to 79% of *P. cepacia* strains have had protease (gelatinase) activity. McKevitt and Woods⁷³ screened 48 *P. cepacia* isolates from patients with CF and found that the majority were proteolytic. Most strains also produced lipase; no exotoxin A- or exotoxin S-like activities were detected. At least one metalloproteinase of *P. cepacia* has been partially characterized.⁷⁴ Studies of proteolytic activity in *P. cepacia* are of particular interest, and proteases are believed to be important in the pathogenesis of *P. aeruginosa* pulmonary infections. Purified proteases from *P. aeruginosa* produce significant pulmonary damage when directly instilled into the lungs of experimental animals. In addition, recent work suggests that proteases of *P. aeruginosa* are the primary virulence factors in acute experimental pneumonia in guinea pigs.⁷⁵

Some strains of *P. cepacia* are capable of synthesizing phenazine pigments similar to those of *P. aeruginosa*.⁷⁶ The conditions for optimal pigment synthesis and the frequency of this trait among clinical strains are not known, but it is interesting to speculate on the extent to which these pigments may inhibit lymphocyte proliferation, as is seen with *P. aeruginosa*.⁷⁷

Despite the in vitro demonstration of these virulence properties, *P. cepacia* has little virulence in animal models. Using a guinea pig model of *Pseudomonas* pneumonia in which intratracheal inoculation of 10⁷ colony-forming units of *P. aeruginosa* reliably produces acute severe, fatal necrotizing pneumonia,⁷⁸ greater inocula of *P. cepacia* were required to produce even mild disease. An isolate from a patient who died of necrotizing *P. cepacia* pneumonia was no more virulent in this model than a strain from a colonized patient (J. Pennington, personal communication). In preliminary experiments (data not shown), we have seen persistence of *P. cepacia* for at least 2 weeks in a rat model of chronic pulmonary infection.⁷⁹ Pathologic changes were similar to those observed using *P. aeruginosa*, and no animals died (data not shown). *P. cepacia* is considerably less virulent than *P. aeruginosa* in the burned-mouse model, whether the organisms are injected intraperitoneally or inoculated onto the burned skin.⁸⁰ However, a highly proteolytic isolate from a patient with CF was found in cultures of the liver 5 days after inoculation of the burn wound, suggesting that bacteremia had occurred. A recent report by Brauner et al.²⁶ may provide a clinical corollary to this observation. Two cases

of *P. cepacia* bacteremia were noted subsequent to burn wound colonization, and strains from both patients were gelatinolytic.

Considerably more work will be needed to determine how *P. cepacia* produces infections in humans. Because *P. cepacia* infections can no longer be considered rare and are very difficult to treat, especially in patients with CF, there is an urgent need for further research.

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The identification of *Pseudomonas cepacia* and its occurrence in clinical material

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During the 19-year period ending December 1984, 4840 strains of Gram-negative non-fermentative bacteria were submitted to the National Collection of Type Cultures for identification. Of these, 195 strains (4.0% of the total) were identified as *Pseudomonas cepacia* which demonstrates both that the species is regularly encountered in clinical material in the UK and that several laboratories have experienced difficulty in identifying the organism. The sources from which the 195 strains were isolated are reported and also the characteristics by which the species may be recognized. The clinical significance of *Ps. cepacia* is reviewed, and the resistance of this species to disinfectants and antimicrobial agents commonly used to treat *Pseudomonas* infections is discussed to underline the necessity for the precise identification of *Ps. cepacia*.

Pseudomonas cepacia, a phytopathogenic species, was originally described by Burkholder (1950). Ballard *et al.* (1970) considered that *Ps. cepacia* was an earlier synonym of '*Ps. multivorans*'. Stanier *et al.* (1966), and as such had nomenclatural priority. There was no phenotypic distinction between *Ps. cepacia* and '*Ps. multivorans*' and although DNA-DNA hybridization experiments supported this view (Ballard *et al.* 1970), a later numerical taxonomic analysis (Sneath *et al.* 1981) of the phenotypic data of Stanier *et al.* (1966) indicated the possibility of some differences between *Ps. cepacia* and '*Ps. multivorans*'. However, all other workers have noted the similarity between *Ps. cepacia* and '*Ps. multivorans*' (e.g. Sands *et al.* 1970) and between these species and strains of the EO-1 group 'eugonic oxidizers, group 1' (King 1964), later called '*Ps. kingii*' by Jonsson (1970; see also Gilardi 1970, 1971; Mackel 1970; Rickett & Pedersen 1970a). Snell *et al.* (1972) established '*Ps. kingii*' (EO-1) as another synonym of *Ps. cepacia* whilst Samuels *et al.* (1973) and Sinsabaugh & Howard (1975) confirmed the synonymy of '*Ps. kingii*' with '*Ps. multivorans*'. Gonzalez & Vidaver (1979) studied

strains of clinical and plant origin and their results also supported the synonymy of *Ps. cepacia*, '*Ps. kingii*' and '*Ps. multivorans*'. *Pseudomonas cepacia* is the correct name nomenclaturally (Palleroni & Holmes 1981).

Pseudomonas cepacia has been reported with increasing frequency in clinical material by workers in the USA and the UK, and with less frequency elsewhere (Table 1). Strains have been isolated from a wide variety of human clinical sources (Table 1) and several reports have appeared detailing the rôle of this organism as the responsible agent in outbreaks of nosocomial infections. In many cases contamination of aqueous topical anaesthetics, disinfectants or irrigating solutions was implicated (Table 1). *Pseudomonas cepacia* is generally regarded as a low grade pathogen (e.g. Hardy *et al.* 1970) but its ability to contaminate and perhaps grow in certain disinfectants in common use in hospitals (e.g. Bassett *et al.* 1970), as well as to grow in deionized or distilled water (Gelbart *et al.* 1975, 1976), makes it a potentially dangerous organism. True infection is seen occasionally (e.g. Bassett *et al.* 1970) but the rôle of the organism has been questioned (Anon. 1970).

Table 1. Summary of previous reports on identification and incidence of *Pseudomonas cepacia*

Country of origin of report	Reference	Common sources of isolates reported (where appropriate)
USA	Sorrell & White (1953)	Blood (endocarditis)
	Schiff <i>et al.</i> (1961)	Blood (endocarditis)
	King (1964, 1967)	Urine, blood, respiratory tract
	Dailey & Benner (1968)	Respiratory tract
	Hardy <i>et al.</i> (1970)	Urine*
	Pickett & Pedersen (1970a, b)	
	Pedersen <i>et al.</i> (1970)	Respiratory tract, blood
	Mackel (1970)	Urinary catheter kits
	Gilardi (1970, 1971, 1972, 1978)	
	Ederer & Matsen (1970)	
	Hugh (1970)	
	Seligman <i>et al.</i> (1971)	Blood (endocarditis)
	Taplin <i>et al.</i> (1971)	Wounds (toe webs)
	Anon. (1972)	Predominantly urine*
	Dixon (1972)	Predominantly urine*
	Ederer & Matsen (1972)	Urine, respiratory tract, wounds, blood
	Gilardi (1972)	Wound, blood, urine
	Schaffner <i>et al.</i> (1973)	Respiratory tract†
	Weinstein <i>et al.</i> (1973)	Respiratory tract
	Dunne <i>et al.</i> (1973)	Blood†
	Anon. (1973)	Blood†
	Meyer (1973)	Blood
	Hamilton <i>et al.</i> (1973)	Blood (endocarditis)
	Rahal <i>et al.</i> (1973)	Blood (endocarditis)
	Neu <i>et al.</i> (1973)	Blood (endocarditis)
	von Gravenitz (1973)	
	Anon. (1974)	Various*
	McKinley & McCroan (1974)	Blood*
	Denney <i>et al.</i> (1975)	Respiratory tract
	Bottom <i>et al.</i> (1975)	
	Noriega <i>et al.</i> (1975)	Blood (endocarditis)
	Feeley <i>et al.</i> (1975)	Respiratory tract
	Matsen (1975)	Predominantly respiratory tract and urine
	Cabrera & Drake (1975)	Blood†
	Gelbart <i>et al.</i> (1975, 1976)	Nebulizer water reservoirs
	Rapkin (1976)	Various†
	Weinstein <i>et al.</i> (1976)	Blood*
	Kaslow <i>et al.</i> (1976)	Blood*
	Frank & Schaffner (1976)	Blood and intravenous fluid*
	Sieber & Fulginiti (1976)	Respiratory tract
	Dixon <i>et al.</i> (1976)	Various*
	Kuehnel & Lundh (1976)	Blood*
	Darby (1976)	Ventricular fluid
	Mandell <i>et al.</i> (1977)	Blood (endocarditis)
	Poe <i>et al.</i> (1977)	Respiratory tract†
	Steere <i>et al.</i> (1977)	Blood†
	Kothari <i>et al.</i> (1977)	Synovial fluid
	Blessing <i>et al.</i> (1979)	Respiratory tract (cystic fibrosis)
	Rhame <i>et al.</i> (1979a, b)	Blood†
	Rosenstein & Hall (1980)	Blood and respiratory tract (cystic fibrosis)
	Berkelman <i>et al.</i> (1981)	Blood*
	Martone <i>et al.</i> (1981)	Various†
	Craven <i>et al.</i> (1981)	Blood*
	Rhame <i>et al.</i> (1981)	Respiratory tract†
	Schmidt <i>et al.</i> (1981)	Respiratory tract
	Rhame & McCullough (1982)	Blood†
	John & Twitty (1982)	Urine*
	Anon. (1982)	
	Berkelman <i>et al.</i> (1982)	Peritoneal fluid

(continued)

Country of origin of report	
	Decicco
	Lybarger
	Gilardi
	Mackel
	Gilligan
	Rutala
	Styrt &
	Smith <i>et al.</i>
	Tablan
	Thomas
	Clegg <i>et al.</i>
UK	Mitchell
	Burdon
	Bassett
	Phillips
	Speller
	Snell <i>et al.</i>
	Speller
	Bassett
	Roberts
	Henders
	Stirland
	King &
	Pallent
	Nakhla
Belgium	DeMol
	Zech
	Yourass
Australia	Juffs
	Morris
	Guinness
	Webbing
France	Richard
	Monteil
Canada	Randall
	Gold <i>et al.</i>
	Isles <i>et al.</i>
	McKevitt
	Conly <i>et al.</i>
Switzerland	Pappalard
Chile	Fernand
Denmark	Bremmel
Denmark and Holland	Borghen
	Siboni <i>et al.</i>
Israel	Sobel <i>et al.</i>
Japan	Yabuuchi
Sweden	Brauner
Trinidad	Morris &

* Source traced to contaminated air coils, pressure transducers and urinary catheters.
† Source traced to contaminated air. The isolate described by Sorrell & King (1964) was established later (Hardy *et al.* 1970).
The isolate described by Schiff *et al.* (1961) was established later (Hardy *et al.* 1970).
The strains of Morris & Roberts (1970) description of '*Ps. multivorans*' (Stanier 1970) and several of the cases described by P. Haigh (1984) were caused by contamin-

Table 1 (continued)

Country of origin of report	Reference	Common sources of isolates reported (where appropriate)
	Decicco <i>et al.</i> (1982)	Nasal sprays
	Lybarger (1982)	Urine
	Gilardi (1983)	
	Mackenzie & Gilligan (1983)	Respiratory tract (cystic fibrosis)
	Gilligan & Schidlow (1984)	Respiratory tract (cystic fibrosis)
	Rutala <i>et al.</i> (1984)	Blood†
	Styrt & Klempner (1984)	Respiratory tract
	Smith <i>et al.</i> (1985)	Bone
	Tablan <i>et al.</i> (1985)	Respiratory tract (cystic fibrosis)
	Thomassen <i>et al.</i> (1985)	Respiratory tract (cystic fibrosis)
	Clegg <i>et al.</i> (1986)	
	Mitchell & Hayward (1966)	Urine*
	Burdon & Whitby (1967)	Contaminated disinfectants
	Bassett <i>et al.</i> (1970)	Wounds*
	Phillips <i>et al.</i> (1971)	Blood†
	Speller <i>et al.</i> (1971)	Urine, blood, wounds*
	Snell <i>et al.</i> (1972)	
	Speller (1973)	Blood (endocarditis)*
	Bassett <i>et al.</i> (1973)	Blood*
	Roberts & Speller (1973)	Urine
	Henderson & Byatt (1974)	Vagina, urine, incubator humidifier water*
	Stirland & Tooth (1976)	Wound, central venous pressure line*
	King & Phillips (1978)	
	Pallent <i>et al.</i> (1983)	
	Nakhla & Haigh (1984)	Blood*, †
	DeMol (1979)	Blood*, †
	Zech (1979)	Various*, †
	Yourassowsky <i>et al.</i> (1979)	Blood†
	Juffs (1973)	Milk
	Morris <i>et al.</i> (1976)	Contaminated disinfectant
	Guinness & Levey (1976)	Predominantly urine and blood*, †
	Webling (1978)	Ventricular fluid
	Richard <i>et al.</i> (1981)	
	Monteil <i>et al.</i> (1981)	Respiratory tract, respirator humidifier water
	Randall (1980)	Various, predominantly urine*, †
	Gold <i>et al.</i> (1983)	Sputum (cystic fibrosis)
	Isles <i>et al.</i> (1984)	Sputum (cystic fibrosis)
	McKevitt & Woods (1984)	Sputum (cystic fibrosis)
	Conly <i>et al.</i> (1986)	Respiratory tract†
	Pappalardo <i>et al.</i> (1980)	Blood
	Fernandez & Otth (1972)	Blood, cerebrospinal fluid, incubator water
	Bremmelgaard (1975)	
	Borghans <i>et al.</i> (1979)	Blood†
	Siboni <i>et al.</i> (1979)	Blood†
	Sobel <i>et al.</i> (1982)	Predominantly urinary tract*
	Yabuuchi <i>et al.</i> (1970)	Blood
	Brauner <i>et al.</i> (1985)	Blood
	Morris & Roberts (1959)	Soil, river water

* Source traced to contaminated antiseptic, disinfectant, etc., in some cases via such sources as haemodialysis coils, pressure transducers and urinary catheter kits.

† Source traced to contaminated anaesthetics, saline, distilled water, etc.

The isolate described by Sorrell & White (1953) was reported as 'a variant of the genus *Herellea*' but its true identity was established later (Hardy *et al.* 1970).

The isolate described by Schiff *et al.* (1961) was identified as '*Flavobacterium*' but its true identity was established later (Hardy *et al.* 1970).

The strains of Morris & Roberts (1959) were not described as *Ps. cepacia* but were included in the original description of '*Ps. multivorans*' (Stanier *et al.* 1966).

Several of the cases described by Phillips *et al.* (1971) and by Zech (1979), and those described by Nakhla & Haigh (1984) were caused by contaminated blood pressure monitoring equipment.

(continued)

Over a 19 year period, up to December 1984, 4840 strains of Gram-negative non-fermentative bacteria were submitted to the National Collection of Type Cultures (NCTC) for identification. Of these, 195 strains (4.0% of the total) were identified as *Ps. cepacia* which suggests both that the organism is not uncommon in clinical material in the UK and that many laboratories encounter difficulty in identifying the species. The results of biochemical tests carried out on 195 strains of *Ps. cepacia* are therefore reported here.

Materials and Methods

BACTERIAL STRAINS

One hundred and ninety-five strains of *Ps. cepacia* were examined. They comprised 191 field strains isolated from clinical material, principally in the UK, and sent to the NCTC for computer-assisted identification; the other four strains were reference cultures maintained in the NCTC. The sources of the field strains are given in Table 2. The four reference cultures were NCTC 10743 (type strain) and strains NCTC 10661, 10734 and 10744.

BACTERIOLOGICAL STUDIES

The 191 field strains were identified in some cases solely on the test results provided by the sending laboratory, in other cases on the test results obtained in the sending laboratory combined with the results of further tests done at the NCTC and in the remaining cases solely on the test results obtained at the NCTC. Only the biochemical test results obtained at the NCTC are reported, so that for strains identified solely on the test results obtained in the sending laboratory no results are given. In addition, as differ-

ent numbers of tests were carried out on each strain, the number of strains examined in each test is given in Table 3. The method of computer-assisted identification used in the NCTC has been described elsewhere (Wilcox *et al.* 1980). The four reference cultures were examined in all the 68 tests listed in Table 3. The methods used for these tests have been described previously (Holmes *et al.* 1975). The test cultures were incubated at 37°C except where otherwise required by the specification for the test.

Results

The strains were Gram-negative rods generally producing non-pigmented colonies on nutrient agar, but 13 (of 173 tested) were yellow pigmented. The biochemical test results are given in Table 3.

Discussion

The biochemical characteristics of the field strains of *Ps. cepacia* (Table 3) agree very closely with those of the reference cultures of the species (which include the type strain) maintained in the NCTC and with the original description of the species given by Burkholder (1950) except that only 30% of the NCTC strains reduced nitrate and 64% produced urease. The biochemical characteristics of all the strains examined also agree well with those presented by other authors. Polymyxin B, colistin and gentamicin are routinely used in the treatment of pseudomonas infections but these agents are not effective against *Ps. cepacia* *in vitro*. Precise identification of the organism thus becomes important, especially as effective therapy may require prolonged or high doses of antimicrobial agents to effect cure. As with

Table 2. Sources of *Pseudomonas cepacia* field strains studied

Source	No. of strains	Source	No. of strains
Blood	42	Catheter tip	14
Transfusion blood	2	Ear swab	2
Respiratory tract	27	Eye swab	2
(sputum, 11)		Anaesthetic	3
(tracheal aspirate or secretion, 15)		Disinfectant	21
(pleural aspirate, 1)		Hospital environment	3
Respirator	21	Miscellaneous	13
Wound swab	12	Unknown	8
Urine	21		

Test
Acid from ASS
glucose
arabinose
cellobiose
fructose
inositol
mannitol
Acid from 10% glucose
Alkali production on
Christensen's citrate
Catalase production
Growth at 37°C
Oxidative in Hugh &
Leifson oxidation-
fermentation test
Tween 20 hydrolysis
Tween 80 hydrolysis
Acid from ASS
adonitol
glycerol
maltose
sorbitol
trehalose
xylose
Growth at room
temperature
Growth on MacConkey
Growth on β -
hydroxybutyrate
Growth on Simmons'
citrate
Opalescence on
lecithovitellin agar
Acid from ASS
dulcitol
lactose
Tyrosine hydrolysis
Casein digestion
Acid from 10% lactose
Cytochrome oxidase
production
Growth on cetrimide
Poly β -hydroxybutyrate
inclusion granules
Acid from ASS salicin
Motility at room
temperature
Gelatin hydrolysis
(plate method)
Urease production
Acid from ASS sucrose
Lysine decarboxylase
Gelatin hydrolysis
(stab liquefaction)
Ornithine decarboxylase
Growth at 42°C

Table 3. Biochemical characters of *Pseudomonas cepacia*

Test	Results of reference strains (no. positive/ no. tested)	Results of field strains (no. positive/ no. tested)	Percentage positive of all strains	Sign
Acid from ASS	4/4	151/151	100	+
glucose	4/4	151/151	100	+
arabinose	4/4	153/153	100	+
cellobiose	4/4	81/81	100	+
fructose	4/4	128/128	100	+
inositol	4/4	111/111	100	+
mannitol	4/4	73/73	100	+
Acid from 10% glucose	4/4			
Alkali production on				
Christensen's citrate	4/4	128/128	100	+
Catalase production	4/4	167/167	100	+
Growth at 37°C	4/4	162/162	100	+
Oxidative in Hugh &				
Leifson oxidation-				
fermentation test	4/4	162/162	100	+
Tween 20 hydrolysis	4/4	147/147	100	+
Tween 80 hydrolysis	4/4	114/114	100	+
Acid from ASS				
adonitol	4/4	129/131	99	+
glycerol	4/4	113/114	99	+
maltose	4/4	153/155	99	+
sorbitol	4/4	110/111	99	+
trehalose	4/4	146/147	99	+
xylose	4/4	152/153	99	+
Growth at room				
temperature	4/4	162/163	99	+
Growth on MacConkey agar	4/4	129/130	99	+
Growth on β -				
hydroxybutyrate	4/4	150/151	99	+
Growth on Simmons'				
citrate	4/4	156/157	99	+
Opalescence on				
lecithovitellin agar	4/4	79/80	99	+
Acid from ASS				
dulcitol	4/4	136/139	98	+
lactose	4/4	110/113	97	+
Tyrosine hydrolysis	4/4	144/148	97	+
Casein digestion	1/4	80/80	96	+
Acid from 10% lactose	4/4	101/107	95	+
Cytochrome oxidase				
production	4/4	158/166	95	+
Growth on cetrimide	4/4	97/110	89	+
Poly β -hydroxybutyrate				
inclusion granules	1/4	130/145	88	+
Acid from ASS salicin	4/4	96/111	87	+
Motility at room				
temperature	4/4	139/164	85	+
Gelatin hydrolysis				
(plate method)	1/4	122/163	74	d
Urease production	2/4	91/142	64	d
Acid from ASS sucrose	3/4	67/112	60	d
Lysine decarboxylase	3/4	90/168	54	d
Gelatin hydrolysis				
(stab liquefaction)	0/4	57/118	47	d
Ornithine decarboxylase	0/4	64/148	42	d
Growth at 42°C	2/4	49/122	41	d

(continued)

ts were carried out on each of strains examined in each Table 3. The method of identification used in the ascribed elsewhere (Willcox et reference cultures were examined listed in Table 3. The or these tests have been sly (Holmes *et al.* 1975). The e incubated at 37°C except required by the specification

Gram-negative rods generally pigmented colonies on nutrient 73 tested) were yellow pigmental test results are given in

l characteristics of the field *Ps. cepacia* (Table 3) agree very closely the reference cultures of the include the type strain) main- NCTC and with the original he species given by Burkholder hat only 30% of the NCTC l nitrate and 64% produced chemical characteristics of all the d also agree well with those pre- r authors. Polymyxin B, colistin e are routinely used in the treat- monas infections but these effective against *Ps. cepacia* in lentification of the organism thus ortant, especially as effective equire prolonged or high doses of agents to effect cure. As with

ains studied	
ource	No. of strains
tip	14
	2
	2
etic	3
ant	21
environment	3
neous	13
n	8

Table 3 (continued)

Test	Results of reference strains (no. positive/ no. tested)	Results of field strains (no. positive/ no. tested)	Percentage positive of all strains	Sign
β -D-Galactosidase production (ONPG test)	3/4	47/122	40	d
Acid from peptone-water- glucose	1/4	52/133	39	d
Aesculin hydrolysis	4/4	39/108	38	d
Motility at 37°C	2/4	59/161	37	d
Nitrate reduction	1/4	42/140	30	d
Acid from ASS raffinose	4/4	21/105	23	d
KCN tolerance	0/4	28/122	22	d
Malonate utilization	0/4	16/122	13	—
Production of yellow pigment	2/4	11/169	8	—
Gluconate oxidation	0/4	6/122	5	—
Growth at 5°C	0/4	6/121	5	—
Acid from ASS ethanol	0/4	1/150	1	—
rhamnose	1/4	0/110	1	—
Arginine dihydrolase	0/4	1/164	1	—
Pigment on tyrosine agar	0/4	2/149	1	—
Arginine desiminase	0/4	0/113	0	—
Deoxyribonuclease production	0/4	0/78	0	—
Fluorescence on King's B medium	0/4	0/109	0	—
Gas from peptone-water- glucose	0/4	0/132	0	—
Hydrogen sulphide production (lead acetate paper)	0/4	0/156	0	—
Hydrogen sulphide production (triple sugar iron agar)	0/4	0/121	0	—
Indole production	0/4	0/120	0	—
Nitrite reduction	0/4	0/126	0	—
Phenylalanine deamination	0/4	0/120	0	—
Reduction of 0.4% selenite	0/4	0/113	0	—
Starch hydrolysis	0/4	0/80	0	—
3-Ketolactose production	0/4	0/78	0	—

The reference strains were NCTC 10661, 10734, 10743 (type strain) and 10744.

+, 85% or more of strains positive; d, 16–84% of strains positive; —, 15% or less of strains positive; ASS, ammonium salt sugar medium; room temperature, 18°–22°C.

other non-fermentative species, insufficient acid is produced in the control tube of the Møller (1955) decarboxylase medium to turn the medium yellow. The unchanged appearance of the control tube can be mistaken for no growth of the strain under test and consequently the decarboxylase tests may be discarded. Decarboxylation of lysine and of ornithine in some strains can thus be overlooked, but positive results are useful diagnostic characters as in clinical material the only other non-fermenter of

clinical interest that decarboxylates lysine is *Ps. maltophilia* and the only other non-fermenter of clinical interest that decarboxylates ornithine is *Alteromonas putrefaciens*.

Esanu & Schubert (1973) recognized three biovars amongst their *Ps. cepacia* strains and Richard *et al.* (1981) recognized eight. Jonsson (1970) found five serogroups and Montell *et al.* (1981) also found five; O and H serotyping of *Ps. cepacia* has been described by Heidt *et al.* (1983). Govan & Harris (1985) have described

bacteriocin typing of *Ps. cepacia* (1981) described mutants blocked of fructose, mannitol and sorbitol, and the growth of *Ps. cepacia* strains have been described by (1974). Nucleic acid similarities of *Ps. cepacia*, *Ps. mallei* and *Ps. pseudomallei* have been studied by Rogul *et al.* (1975). Petersen (1975) reported the phage sensitivity of *Ps. cepacia* cells following exposure to light; their work suggests a possible role for phage in the contamination in u.v.-treated water.

As well as occurring in soil and surface waters (Morris *et al.* 1959; Tapscott *et al.* 1970), *Ps. cepacia* can also be found in the hospital environment, especially water and surfaces (Bassett *et al.* 1970; Gilardi *et al.* 1971) where it is able to survive disinfectants. *Pseudomonas cepacia* has been found to be inhibited by 0.5% chlorhexidine + 0.5% cetrimide at 1 in 10 dilution, but large inocula survive even 1 in 30 (Bassett *et al.* 1970). Inocula of 10^5 – 10^7 cells/ml have been found in aqueous chlorhexidine alone (Spencer *et al.* 1970) and as many as 10^3 cells/ml have been found in commercial urinary catheter irrigation solutions (0.15% N-alkyl, dimethylbenzylamine hydrochloride in water and phenoxymethyl alcohol (Detergicide; Hardy *et al.* 1970). It has also failed to eradicate this organism in a blood pressure monitoring apparatus contaminated heparinized saline solution (Phillips *et al.* 1971). Nishimura (1984) also noted infections resulting from contaminated blood pressure monitoring apparatus. The source of contamination was deionized water used to prepare disinfecting the transducer cable. *Ps. cepacia* has also been found to be resistant to benzalkonium chloride (Bassett *et al.* 1970) and to the organic preservative thimerosal (Decicco *et al.* 1970) although isopropyl alcohol and benzalkonium chloride are effective (Burdon & Whitby 1971; Nakhla & Haigh 1984). It is important to realize that infections due to this organism often have been initiated by contact with contaminated or antiseptic solutions. This is illustrated in Table 1 where it can be seen that 191 NCTC field isolates of *Ps. cepacia* recovered from disinfectants (Table 1) and *Ps. cepacia* can also grow in

bacteriocin typing of *Ps. cepacia*. Allenza *et al.* (1981) described mutants blocked in utilization of fructose, mannitol and sorbitol. Factors influencing the growth of *Ps. cepacia* in sugar solutions have been described by Carson *et al.* (1974). Nucleic acid similarities among *Ps. cepacia*, *Ps. mallei* and *Ps. pseudomallei* have been studied by Rogul *et al.* (1970). Carson & Petersen (1975) reported the photoreactivation of *Ps. cepacia* cells following exposure to u.v. light; their work suggests a possible source of contamination in u.v.-treated waters.

As well as occurring in soil and natural waters (Morris *et al.* 1959; Taplin *et al.* 1971), *Ps. cepacia* can also be found in the hospital environment, especially water and moist surfaces (Bassett *et al.* 1970; Gilardi 1970; Phillips *et al.* 1971) where it is able to survive certain disinfectants. *Pseudomonas cepacia* has been found to be inhibited by Savlon (0.05% chlorhexidine + 0.5% cetrimide) at a 1 in 320 dilution, but large inocula survived dilutions of even 1 in 30 (Bassett *et al.* 1970). Concentrations of 10^2 – 10^7 cells/ml have been found in 0.02% aqueous chlorhexidine alone (Speller *et al.* 1971) and as many as 10^3 cells/ml have been found in commercial urinary catheter kits containing 0.15% N-alkyl, dimethylbenzyl ammonium chloride in water and phenoxyethoxyethanol (Detergicide; Hardy *et al.* 1970). Detergicide also failed to eradicate this organism from blood pressure monitoring apparatus in which contaminated heparinized saline solutions had been used (Phillips *et al.* 1971). Nakhla & Haigh (1984) also noted infections resulting from contaminated blood pressure monitoring apparatus. The source of contamination was deionized water used to prepare Detergicide for disinfecting the transducer cables. *Pseudomonas cepacia* has also been found to be resistant to benzalkonium chloride (Bassett *et al.* 1970; Gilardi 1970) and to the organomercurial preservative thimerosal (Decicco *et al.* 1982) although isopropyl alcohol and glutaraldehyde are effective (Burdon & Whitby 1967; Phillips *et al.* 1971; Nakhla & Haigh 1984). It is not surprising that infections due to this organism have often been initiated by contaminated disinfectant or antiseptic solutions. This is well illustrated in Table 1 where it can be seen that 21 of the 191 NCTC field isolates of this species were recovered from disinfectants (Table 2). *Pseudomonas cepacia* can also grow in either doubly

deionized or doubly distilled water as well as 5% dextrose or 0.9% saline (Gelbart *et al.* 1975, 1976) but it does not grow in intravenous feeding solutions under the usual conditions. Contamination may be easily overlooked as turbidity is not visible (presumably because of the extremely small size of the cells under sub-optimal conditions) even when the organism is grown in distilled water to a concentration of 10^7 /ml (Carson *et al.* 1973). Infections due to this organism can thus also be initiated by contaminated distilled water or intravenous solutions (Table 1).

Pseudomonas cepacia is more resistant than most other Gram-negative bacteria to the commonly used antimicrobial agent benzalkonium chloride. It is also highly resistant to polymyxin B sulphate, another cationic agent that is effective against most Gram-negative organisms. Both agents are thought to act primarily against the cytoplasmic membrane. However, although the work of Adair *et al.* (1976) suggested the cytoplasmic membrane of *Ps. cepacia* to be resistant to polymyxin B (although not to benzalkonium chloride), Manniello *et al.* (1978) believed that resistance to polymyxin B probably involved more than just a barrier effect. The study of Parmelee & Walker (1979), however, suggested that resistance to aminoglycosides (see below) was at the level of the cytoplasmic membrane. Survival for several years in benzalkonium chloride has been reported by Mathews *et al.* (1975), Adair & Gefitc (1976) and Gefitc *et al.* (1979). The authors of the two latter publications concluded that pharmaceutical solutions containing benzalkonium chloride as an antimicrobial preservative should be formulated without extraneous carbon and nitrogen sources or be preserved with additional antimicrobial agents. Other authors have pointed out (Frank & Schaffner 1976) that in view of the many outbreaks of infection associated with contaminated benzalkonium chloride (Table 1) that such solutions should no longer be used in hospitals. Resistance of *Ps. cepacia* to benzalkonium chloride, as well as to polymyxin B sulphate and chlorhexidine gluconate, has been studied by Richards & Richards (1979). Electron microscopy of the effect of these three agents on the cytoplasmic membrane has been performed by Richards & Cavill (1980). Bassett (1971) reported that while *Ps. cepacia* can survive in Savlon (chlorhexidine gluconate and

cetrimide) diluted with distilled water it cannot do so when hard tap water is used as the diluent; pH thus has an important influence on the effectiveness of such solutions. Borovian (1983) encountered *Ps. cepacia* in a stored product and found the organism to survive and grow in the formulation even though the pH was less than 3.2. She also found that the strain was able to acquire resistance to two unrelated preservative systems. Adherence of the bacterial cells to glass surfaces appears to afford some protection from chlorhexidine (Pallent *et al.* 1981; Hugo *et al.* 1986).

Pseudomonas cepacia may be recovered commonly from blood (Tables 1 and 2). Septicaemia with endocarditis can occur (Table 1) and in the two earliest reports (Sorrell & White 1953; Schiff *et al.* 1961) the patients died; in the former case, infection may have originated from the urinary tract. There have been several reports of the isolation of *Ps. cepacia* from blood following cardiac surgery (Pedersen *et al.* 1970; Phillips *et al.* 1971; Speller *et al.* 1971; McKinley & McCroan 1974; Weinstein *et al.* 1976). The patients usually recovered spontaneously or with antimicrobial therapy if contamination of the blood pressure monitor or of the intravenous catheter had been the cause of the septicaemia. Two of the NCTC field strains from blood are also known to have been recovered from patients following heart surgery. Endocarditis due to *Ps. cepacia* has been reported in narcotic addicts; one case involved a previously implanted Starr-Edwards mitral prosthetic valve and the other a previously normal aortic valve in a patient with sickle cell anaemia; both cases proved fatal (Seligman *et al.* 1971). The organism has also caused cervical osteomyelitis in an intravenous drug abuser (Smith *et al.* 1985). A case of *Ps. cepacia* endocarditis with ecthyma gangrenosum in a heroin addict was described by Mandell *et al.* (1977). *Pseudomonas cepacia* septicaemia has also been described as the cause of death in two burn patients (Yabuuchi *et al.* 1970; Brauner *et al.* 1985), the several antimicrobial agents administered proved ineffective against the organism in one of the cases (Yabuuchi *et al.* 1970). Two patients who died from bacteraemia both had severe underlying disease (Sobel *et al.* 1982); the use of contaminated chlorhexidine was again the vehicle of infection in these two (and several other) patients.

Pseudomonas cepacia is less frequently associated with wounds (Tables 1 and 2) although reports include nine cases of post-operative wound infection caused by local application of a contaminated disinfectant solution (Savlon; Bassett *et al.* 1970). Taplin *et al.* (1971) isolated *Ps. cepacia* from the toeweb of 43/51 (85%) army ranger trainees after swamp training in northern Florida. Within the context of foot rot, jungle rot or swamp rot diseases, the authors maintained that there is a clinical entity, associated with *Ps. cepacia*, involving the toeweb and occasionally the plantar surface of the foot, that is characterized by hyperkeratosis, maceration, and sometimes induration, inflammation and fissuring. Septic arthritis has arisen following intraarticular injection of contaminated methylprednisolone (Kothari *et al.* 1977).

Pseudomonas cepacia is commonly associated with urinary tract infections (Tables 1 and 2) although in instances where these have been due to contaminated catheter irrigation fluid, infections rarely produced symptoms (Hardy *et al.* 1970; Speller *et al.* 1971) and in many patients with positive urine cultures the urine became sterile spontaneously after catheter removal. In patients with symptoms, however, appropriate antimicrobial therapy did not always eradicate the organism from the urine (Mitchell & Hayward 1966; Roberts & Speller 1973). Contaminated urological instruments have been the vehicle of infection in other hospital outbreaks (Dixon 1972; Anon. 1974). Ederer & Matsen (1972) noted that in 37 of 41 patients from whom *Ps. cepacia* was isolated, prior instrumentation or a manipulative procedure at the site of recovery of the organism strongly suggested that most were nosocomial infections.

Strains of *Ps. cepacia* are also recovered from the respiratory tract or associated equipment (Tables 1 and 2) (Gelbart *et al.* 1975, 1976; Blessing *et al.* 1979) but true infection is rare. One case of true infection was that of a man who developed necrotizing pneumonia after cleaning air conditioners for 3 weeks (Dailey & Benner 1968). Bronchial washings and lung tissue cultures grew *Ps. cepacia*. Therapy was effective with high doses of chloramphenicol (12 g daily). Pneumonitis due to *Ps. cepacia* recurred in this patient and he was found to suffer a phagocyte dysfunction (Dennéy *et al.* 1975); temperature returned to normal within 36 h of commencing treatment, again with chlo-

ramphenicol 12 g daily. Lung function was improved in chronic granulomatous disease and the associated syndrome has been described (1975) who described prolonged prophylaxis may well have prevented colonization and further cases of apparent chronic bronchitis have been described (Schmidt *et al.* 1976; Clegg *et al.* 1986; Weinstein *et al.* 1986). A boy following chemotherapy did not show a therapeutic response to chloramphenicol (100 mg/kg). A child developed a lung infection; ultrasonic nebulizer proved ineffective. *Ps. cepacia* (Poe *et al.* 1976) chloramphenicol patient died. An infection complicated by septicaemia was described (1980). In fact, reports of *Ps. cepacia* in patients (Table 1) the recovery of respiratory secretions have been developed medium for recovery of water, solutions by Wu & Thorpe (1984) reported respiratory tract culture of 425 CFU patient. *Ps. cepacia* appeared in the sputum of the patient. Some of the patients for long time periods with no dramatic improvement, others seemed to have rapid deterioration. Most of the infections at the site of colonization it is the organism. Similar findings (1984), Tablan *et al.* (1985). *Pseudomonas cepacia*

frequently associated and 2) although of post-operative al application of a solution (Savlon; al. (1971) isolated s of 43/51 (85%) wamp training in context of foot rot, ases, the authors ical entity, associ- g the toeweb and e of the feet, that tosis, maceration, nflammation and arisen following aminated methyl- 77).

monly associated (Tables 1 and 2) ese have been due gation fluid, infec- ms (Hardy et al. in many patients he urine became heter removal. In ever, appropriate always eradicate ine (Mitchell & ells 1973). Con- nts have been the ospital outbreaks derer & Matsen 41 patients from , prior instrumen- dure at the site of rongly suggested ections.

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chloramphenicol 12 g daily. The phagocyte dysfunction was indistinguishable from that found in chronic granulomatous disease of childhood and the association of *Ps. cepacia* with the latter syndrome has been noted by Bottone et al. (1975) who described three such cases. Prolonged prophylactic and antimicrobial therapy may well have played a significant role in the colonization and infection of these patients. Further cases of pneumonia in children with apparent chronic granulomatous disease have been described (Sieber & Fulginiti 1976; Schmidt et al. 1981; Styrt & Klempner 1984; Clegg et al. 1986). Another case was reported by Weinstein et al. (1973) of pneumonia in a young boy following cardiac surgery; the patient did not show a therapeutic response until his daily chloramphenicol dose was increased to 3 g (100 mg/kg). A diabetic patient with pneumonia developed a lung abscess after therapy with ultrasonic nebulization (the reservoir of the nebulizer proved to be contaminated with *Ps. cepacia* (Poe et al. 1977)), despite treatment with chloramphenicol for an extended period, the patient died. Another case of cystic fibrosis (CF) complicated by *Ps. cepacia* pneumonia and septicaemia was described by Rosenstein & Hall (1980). In fact, recent years have seen increasing reports of *Ps. cepacia* isolation from cystic fibrosis patients (Table 1). An isolation medium for the recovery of this organism from the respiratory secretions of such patients has indeed been developed (Gilligan et al. 1985; a selective medium for recovery of the organism from water, solutions and lotions has been described by Wu & Thompson 1984). Gilligan & Schidlow (1984) reported the organism from respiratory tract cultures of approximately 20% of 425 CF patients. They found that recovery of *Ps. cepacia* appeared to be associated with deterioration of the clinical status of some patients. Some of the patients harboured the organism for long time periods (for 6 years in one patient with no dramatic change in his disease) but others seemed to show a progressive and rather rapid deterioration in their clinical condition. Most of the individuals had rather severe lung disease at the time of colonization and once colonized it is almost impossible to eradicate the organism (Gilligan & Schidlow 1984). Similar findings were reported by Isles et al. (1984), Tablan et al. (1985) and by Thomassen et al. (1985). *Pseudomonas cepacia* strains from CF

patients appear to be particularly virulent (Montie et al. 1985) and this is possibly associated with the ability of such strains to produce pyochelin (Sokol 1986).

The distribution by source of the strains submitted to the NCTC for identification (Table 2) reflects their incidence in clinical specimens (Table 1). Unfortunately, few clinical details were received with the strains so the literature has to be relied upon for assessing the clinical significance of the organism. However, three of the NCTC strains may have been clinically significant. One strain was isolated on three separate occasions from a thoracic wound following surgery to correct a ventricular septal defect. Another strain was isolated from an intravenous catheter tip and from subsequent blood culture of a 23-year-old man with clinical septicaemia following pulmonary embolus and pneumonia. He had been admitted as a road traffic accident casualty with a wedge fracture of the third lumbar vertebra and lacerations to the legs. *Pseudomonas cepacia* was not isolated from wound swabs, sputum or urine or from aqueous chlorhexidine plus cetrimide used to disinfect wound tissue. The third strain was isolated in pure culture from an infected dog bite wound. In general, *Ps. cepacia* has a low order of pathogenicity. In the outbreak of infection caused by contaminated anaesthetics described by Schaffner et al. (1973) clinical disease did not develop in any patient despite the introduction of large inocula, in several cases more than 10 ml of anaesthetic containing 10^5 – 10^{10} bacteria/ml. Similarly, Leyden et al. (1980) reported that an inoculum in excess of 10^5 cells is required to produce cutaneous damage even on scarified skin. It can be seen that *Ps. cepacia* poses a serious threat to the patient in relatively few situations despite the fact that few antimicrobial agents are regularly effective.

Strains of *Ps. cepacia* are generally resistant to ampicillin, carbenicillin, cephalothin, colistin, gentamicin, kanamycin, neomycin, polymyxin B, streptomycin and tetracycline (Moody et al. 1972; Matsen 1975). In particular, resistance to amikacin, gentamicin and tobramycin has been studied by Moellering et al. (1977). Distinct resistance patterns have been observed amongst isolates of the species (Moody et al. 1972). Chloramphenicol has been used in systemic treatment (Dailey & Benner 1968; Hardy et al. 1970) and the combination of trimethoprim with

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EXPRESS MAIL NO.: EV 473 971 532 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Casida *et al.*

Confirmation No.: 3630

Application No.: 10/776,767

Art Unit: 1651

Filed: February 10, 2004

Examiner: Afremova, Vera

For: NON-OBLIGATE PREDATORY
BACTERIUM *BURKHOLDERIA*
CASIDAE AND USES THEREOF

Attorney Docket No.: 8014-020-999

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In accordance with the continuing duty of disclosure imposed by 37 C.F.R. § 1.56 to inform the United States Patent and Trademark Office of all references coming to the attention of each individual associated with the filing or prosecution of the subject application which are or may be material to the patentability of any claim of the application, Applicants hereby direct the Examiner's attention to references C01-C21 listed on the accompanying List of References Cited by Applicant. Copies of references C01-C21 are submitted herewith.

Identification of the listed references is not to be construed as an admission of Applicants that such references are available as "prior art" against the subject application.

Applicants respectfully request that the Examiner review the listed references and that the references be made of record in the file history of the application.

Appl. No. 10/776,767
Attorney Docket No. 8014-020-999
Reply dated June 15, 2007
Reply to Office Action mailed Feb. 5, 2007

Pursuant to 37 C.F.R. § 1.97(c), since this Supplemental Information Disclosure Statement is being filed after the mailing date of a first Office Action on the merits, but before the mailing date of a final action under 37 C.F.R. § 1.311 or an action that otherwise closes prosecution in this application, it is estimated that a fee of **\$180.00** as set forth in 37 C.F.R. § 1.17(p) is due for this submission. Please charge the required fee to Jones Day Deposit Account No. 50-3013. A copy of this sheet is enclosed for accounting purposes.

Respectfully submitted,

Date: June 15, 2007

Laura A. Coruzzi by Ann W. Chen
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Enclosures



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LIST OF REFERENCES CITED BY APPLICANT

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APPLICANT

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U.S. PATENT DOCUMENTS

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	C01	W. ROBERT BAILEY & ELVYN G. SCOTT, Diagnostic Microbiology (4th ed. 1974), C. V. Mosby Company, St. Louis, MO, Chapter 34, "Determination of susceptibility of bacteria to antimicrobial agents."
	C02	BLOUSE <i>et al.</i> , "Colonization and infection of newborn infants caused by bacteriophage-group II <i>Staphylococcus aureus</i> strains," J Clin Microbiol. 1979 Oct;10(4):604-6.
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Colonization and Infection of Newborn Infants Caused by Bacteriophage-Group II *Staphylococcus aureus* Strains

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A staphylococcal strain which exhibited weak lytic reaction with group II phages was isolated from a newborn infant with a skin infection. Subsequent investigations established that this weakly reacting strain was responsible for an endemic level of infection and colonization within the hospital nursery. The use of consistently appearing weak lytic reactions in the evaluation of this episode is described.

Staphylococcus disease in hospital nurseries is a continuing concern to those responsible for infection control. The approach used in monitoring such infections includes mandatory culture of all infections, e.g., wounds, rash, pustules, etc., and routine culture of the cord and external nares. *Staphylococcus aureus* strains recovered

ically in a nursery and recovered sporadically from a series of infections and colonizations of newborn infants. The nursery had 31 operating bassinets with an average of 71 admissions per month during the period of study. This report briefly describes the clinical-epidemiological and laboratory features of this episode.

TABLE 1. Occurrence and phage type of group II *S. aureus* strains among newborn infants, U.S. Air Force Medical Center, Keesler Air Force Base, Miss. (January to August 1978)

Patient	Specimen source	Culture date	Phage type	
			RTD	100 × RTD
A	Pustular rash	9 February	NT ^a	3A ⁺ /3C ⁺
B	Blood	23 March	NT	3C ⁺
	Skin lesions	27 March	NT	3C ⁺
C	Nasal (colonization)	3 April	NT	3C ⁺
	Bullous lesions	3 April	NT	3C ⁺
D	Skin lesions	19 April	NT	3C ⁺ /55 ⁺
E	Bullous lesions	28 April	NT	3C ⁺ /55 ⁺
	Bullous lesions	19 April	NT	3C ⁺
F	Chest tube drainage (colonization)	5 May	NT	3C ⁺⁺
G	Nasal (colonization)	23 June	NT	3C ⁺
H	Cord (colonization)	28 June	NT	3C ⁺
I	Nasal (colonization)	28 June	NT	3C ⁺
	Bullous lesions	28 June	NT	3C ⁺
J	Eye drainage	12 July	NT	3C ⁺
K	Nasal lesions	9 August	3A ⁺	3A ⁺ /3C ⁺⁺

^a NT, Nontypable

from such infections are usually tested for antimicrobial susceptibility and are often phage typed as part of a surveillance program. At a large Air Force medical center, we encountered an unusual situation in which a "weakly typing" group II *S. aureus* strain was maintained endem-

As a part of the hospital infection control program at the U.S. Air Force Medical Center, Keesler Air Force Base, Miss., *S. aureus* isolates recovered from hospitalized patients with infections are periodically forwarded to the U.S. Air Force School of Aerospace Medicine's Epidemiology Division laboratory for phage typing. In early February, 1978, a female infant was seen

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at the Pediatric Clinic with a pustular rash over the lower abdomen and inner right thigh. Bacteriological examination of the pustular exudate was positive for *S. aureus*. The infant was treated with oral dicloxacillin with good clinical response. This infant had had an uncomplicated nursery stay (22 to 28 January) without apparent cord colonization with *S. aureus*. Phage typing of the isolate—with the currently accepted international set of typing phages 3A, 3C, 6, 29, 42E, 47, 52, 52A, 53, 54, 55, 71, 75, 77, 79, 80, 81, 83A, 84, 85, 94, 95, and 96 at routine test dilution (RTD) and 100× RTD (2)—showed no lytic reaction at RTD, and only a weak 3A⁺/3C⁺ lytic reaction at 100× RTD. A review of phage typing results of *S. aureus* isolates cultured from newborns during January and February did not indicate any similar phage group II patterns.

The occurrence of this weakly reactive group II phage type was not documented again until 27 March, when an infant, born by repeat caesarean section on 23 March, developed erythematous, crusted lesions on the thigh. *S. aureus* was recovered from these lesions and blood cultures taken shortly after delivery; both isolates were characterized by weak lytic reactions with typing phage 3C. During the period February to August, a total of eight infants, including the index case, developed infections (seven involving skin lesions and one involving eye infection) attributable to the group II phage type, whereas three infants were colonized but not infected (Table 1). Four of these infections and one apparent colonization without infection were clustered during an 8-week period, between 23 March and 15 May, with no more than 1 week separating infections. Infected infants often had overlapping nursery stays which would account, in part, for the maintenance and endemicity of the group II strain. It is not unlikely that one or more medical staff personnel may have been colonized with this strain and served as a reservoir for dissemination as well.

The observation that group II staphylococci are frequently associated with skin infections in infants has been well documented (1, 3, 4). The clinical severity of these infections can range from a localized bullous impetigo to a more widespread involvement with or without exfoliation. The value of weak typing reactions in evaluating strains which exhibit apparently dissimilar strong reactions has been described (2). The strains encountered at the Keesler Medical Center were unusual, in that, with one exception, strongly reacting group II strains were never associated with endemic infection or colonization during this period. However, the group II strains recovered consistently produced weak

(± to +) reactions when tested at 100× RTD. It should be noted that propagating strain controls were included with each series of strains for phage typing to determine whether the RTD was satisfactory for use. Additionally, phage stocks used for the preparation of test dilutions (RTD) were as received from the Center for Disease Control, Atlanta, Ga., and had not been further propagated in our laboratory. A review of phage typing reactions of strains submitted from more than 35 Air Force hospitals in the United States indicated that the strain had been encountered only occasionally. Fortunately, during the episode reported here, infections were localized for the most part, and all infants responded readily to treatment with intravenous

TABLE 2. Occurrence of other *S. aureus* phage types among newborn infants, U.S. Air Force Medical Center, Keesler Air Force Base, Miss. (January to August 1978)

Patient	Specimen source	Culture date	Phage type	
			RTD	100 × RTD
1	Nose	3 January	6/47/54/75	
2	Nose	13 January	NT ^a	6/42E/47/53/54/75
3	Eye drainage	16 January	29/52/52A/80/55/71	
3	Cord	16 January	29/52/80/55/71	
4	Cord	16 January	NT	NT
5	Nose	23 January	96	
6	Nose	23 January	54/75	
7	Nose	23 January	54/75	
8	Nose	23 January	47/54/75	
9	Nose	30 January	96	
10	Nose	30 January	NT	NT
11	Nose	23 March	NT	NT
12	Nose	3 April	6/47/75/83A	
13	Nose	10 April	94/96	
14	Nose	10 April	NT	NT
15	Unknown	10 April	NT	55
16	Unknown	10 April	95	
17	Cord	24 April	NT	NT
18	Cord	24 April	NT	NT
18	Nose	24 April	NT	NT
19	Wound drainage	24 April	95	
20	Cord	24 April	6/47/75/83A	
21	Nose	11 May	NT	NT
22	Nose	22 May	96	
23	Nose	22 May	96	
24	Nose	6 June	NT	NT
25	Nose	18 July	NT	NT
26	Nose	31 July	NT	NT
27	Wound	31 July	47/54/85	
28	Nose	31 July	NT	NT
29	Nose	17 August	47/54/75	
30	Unknown	17 August	47/54/75	
31	Nose	28 August	NT	NT
32	Nose	28 August	NT	NT
33	Unknown	28 August	NT	NT
34	Nose	28 August	47/54/75	

^a NT, Nontypable.

methicillin, usually followed by a 10-day regimen of oral dicloxacillin. No deaths were recorded. Although no new control measures were instituted during the period of these infections, increased bacteriological monitoring of *S. aureus* colonization among newborn infants was followed, and hand washing by personnel between each infant contact was reinforced. Other *S. aureus* phage types, isolated from newborn infants, were not related to the group II strains (Table 2). There was no unusual increase in the *S. aureus* colonization rate throughout this time.

This experience emphasizes the importance that *S. aureus* infections in the newborn nursery should be monitored, with special emphasis placed on group II strains such as the one de-

scribed. The laboratory should make available comprehensive phage typing results recording strong and weak lytic reactions for all strains found nontypable at RTD.

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DIAGNOSTIC MICROBIOLOGY

A textbook for the isolation and
identification of pathogenic
microorganisms

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34 Determination of susceptibility of bacteria to antimicrobial agents

According to Isenberg,¹² the value of the clinical laboratory can be measured only by the significance of the guidance it gives the practicing physician in the treatment of his patients. In no other area of clinical microbiology does this statement become more pertinent than in the testing of clinical isolates for their susceptibility to antimicrobial agents. With the increasing number of these agents at the physician's disposal and the changing pattern of resistance and susceptibility among bacteria—particularly the gram-negative enteric bacilli—the clinician must rely more and more upon sensitivity testing to guide his selection of appropriate drugs or alter an already imposed regimen. Therefore, to a large extent, a laboratory report showing susceptibility or resistance to a particular antimicrobial agent becomes an endorsement of its usefulness or withdrawal.

Since the microbiologist must become an adviser to the physician regarding proper antimicrobial therapy, it follows, therefore, that he must maintain (1) a high level of accuracy in his testing procedures, (2) a high degree of reproducibility for the results, and (3) a good correlation of his results with the clinical response.¹² Only through close cooperation and exchange of information between the laboratory staff and the clinician can the best possible management of an infectious process be achieved.

The principal methods presently used

by the laboratory to determine susceptibility of a microorganism to an antibiotic include the **dilution tests**, such as the broth tube and agar plate dilution procedures, and the **agar diffusion test**, utilizing antibiotic-impregnated discs. Each method has its advantages and its limitations, and these must be understood and appreciated in order to obtain maximum usefulness of the results. Since there is a place for all of these methods in the clinical laboratory, the procedures and directions for the use of each will be described in detail.

In the interpretation of any in vitro susceptibility tests, it is well to remember that they are essentially **artificial measurements**; the data yielded by them give only the approximate range of effective inhibitory action against the microorganisms. The only absolute criterion of microbial response to antibiotics is the **clinical response** of the patient when adequate dosage of the appropriate antibiotic is administered.

BROTH TUBE DILUTION METHOD FOR DETERMINING SUSCEPTIBILITY TO ANTIBIOTICS

In the broth tube dilution method for determining the susceptibility of an organism to antibiotics, specific amounts of the antibiotic, prepared in decreasing concentration in broth by the serial dilution technique, are inoculated with a culture of the bacterium to be tested. The susceptibility

of the organisms is determined, after a suitable period of incubation, by microscopic observation of the presence or absence of growth in the varying concentrations of the antimicrobial agent. This bacteriostatic end-point value is known as the **minimal inhibitory concentration** (MIC). With minor additions, the technique can be adapted to the determination of bactericidal levels of the antibiotic—the **minimal bactericidal concentration** (MBC); this is discussed at length in a further section.

A number of factors must be considered in establishing the procedures and in evaluating the results of these tests.⁸ They include the following: (1) the medium in which the tests are performed, (2) the stability of the antibiotic, (3) the size of the inoculum, (4) the rate of growth of the organism, and (5) the period of incubation of the tests. Any variation in one or more of these factors may influence the tests, and the results obtained by one procedure may not agree with those arrived at by a slightly different method.⁵ However, if a **standard procedure using only pure cultures** is adopted and strictly adhered to, reproducible results can usually be obtained and the reports from a given laboratory can be readily interpreted by the clinical staff.

The test tube serial dilution method gives a fairly accurate determination of susceptibility to measured amounts (either units or micrograms) of the antibiotic. It is a time-consuming and expensive procedure, however, especially when the clinician wants to know the susceptibility of an organism to a number of antibiotics. For this reason its use may well be restricted to special cases when quantitative results may be of value. In any event, it is strongly recommended that all clinical laboratories should be prepared to offer this service to the clinician, either directly or through a referral laboratory.

The serial dilution method may be recommended for determining the susceptibility of organisms isolated in the following instances: (1) from blood cultures, (2)

from patients who fail to respond to apparently adequate therapy, and (3) from patients who relapse while undergoing such therapy. The study of organisms from the third instance usually involves determination of any increase in resistance of subsequent isolations and requires special methods.

Routine procedure for serial dilution tests

Preparation of stock solution of antibiotics

Stock solutions of antibiotics are prepared from concentrated, dehydrated sterile material of known potency that may be obtained from the pharmaceutical manufacturer. Generally, they are prepared in concentrations of 1,000 $\mu\text{g./ml.}$, using phosphate buffer or Mueller-Hinton broth as the diluent and are tubed in 1-ml. amounts in screw-capped vials.

When stored in the frozen state at -20°C. , these antibiotics will remain stable for at least 8 weeks*; when refrigerated at 5°C. , they show no appreciable loss of potency in 1 week. Any unused thawed solutions of antibiotics should be discarded; each aliquot should be sufficient for 1 day's use only and should not be refrozen.

Table 34-1 is provided as a guide to the preparation of stock solutions of the most frequently used antimicrobial agents.

Selection of media

The fluid media in which the tube dilution sensitivity tests are carried out must be the kind that will support optimal, rapid growth of the test organism in pure culture. A broth medium that will support the growth of pneumococci and streptococci without the addition of serum or blood is preferable, since the addition of such enrichment adds another variable to the test and may influence the results. Trypticase soy broth, or preferably Mueller-Hinton broth† is recommended for sensitivity tests with the following ex-

*Ampicillin requires storage at -60° to -70°C. to prevent loss of potency.

†This medium appears to be low in tetracycline and sulfonamide inhibitors and shows good batch-to-batch consistency.

Table 34-1. Procedures for preparing stock solutions*

ANTIBIOTIC	MANUFACTURER	METHOD OF PREPARATION
Ampicillin	Bristol-Myers Co.	Weigh out material and multiply by "activity standard" provided by manufacturer.† Add 0.1 ml. of pH 8.0 phosphate buffer to dissolve; dilute with pH 6.0 phosphate buffer.
Penicillin G	Eli Lilly & Co.	Add 60 ml. of water to a vial containing 1 million units or 600,000 µg.; this makes a stock solution of 10,000 µg./ml.
Methicillin	Bristol-Myers Co.	Weigh out material and multiply by "activity standard" from manufacturer†; dilute with pH 6.0 phosphate buffer.
Oxacillin	Bristol-Myers Co.	Add 16 ml. of water to a vial to give 1,000 µg./ml.
Cephalothin	Eli Lilly & Co.	Weigh out material and multiply by the "activity standard," from the manufacturer†; dilute with pH 6.0 phosphate buffer.
Cephaloridine	Eli Lilly & Co.	Weigh out exactly 30 mg., add 30 ml. of pH 6.0 phosphate buffer to give 1,000 µg./ml.
Carbenicillin	Beecham-Massengill Pharmaceuticals	Add 10 ml. of water to a vial containing 1 gm. of drug, then dilute 1:100 to give 1,000 µg./ml.
Tetracycline	Pfizer Lab.	Add 20 ml. of water to a vial that contains 20 mg. of drug, to give 1,000 µg./ml.
Chloramphenicol	Parke, Davis & Co.	Weigh out exactly 50 mg., add 1 ml. of ethyl alcohol to dissolve drug and sufficient water to 50 ml. This gives a concentration of 1,000 µg./ml.
Erythromycin	Abbott Laboratories	Weigh out material and multiply by the "activity standard" from the manufacturer.† Dissolve in 1-2 ml. alcohol and add water to a final concentration of 1,000 µg./ml.
Lincomycin	The Upjohn Co.	Add 20 ml. water to a vial to make 1,000 µg./ml.
Clindamycin	The Upjohn Co.	Add 15 ml. of water to vial containing 150 mg. of drug, dilute 1:10 to give 1,000 µg./ml.
Kanamycin	Bristol-Myers Co.	Weigh out exactly 30 mg., add 30 ml. water to give 1,000 µg./ml. Agent is unstable in acid range.
Gentamicin	Schering Corp.	Weigh out material and multiply by "activity standard" from manufacturer,† dilute with water to give 1,000 µg./ml.
Polymyxin B	Burroughs Wellcome & Co.	Add 5 ml. water to a vial containing 50 µg., to give 10,000 µg./ml.; dilute to desired concentration.
Colistin (polymyxin E)	Warner-Chilcott Lab.	Weigh out material and multiply by "activity standard" from manufacturer,† dilute with water to desired concentration.
Bacitracin	The Upjohn Co.	Add 10 ml. of water to a vial containing 10,000 units, to give 1,000 units/ml.
Nitrofurantoin	Eaton Labs., Inc.	Weigh out 120 mg. of drug and transfer to a 50-ml. flask containing 4.0 ml. of dimethyl formamide. Heat in 56° C. water bath with shaking to dissolve. This solution contains 30 mg./ml.
Nalidixic acid	Winthrop Labs.	Weigh out approx. 30 mg., add 2 ml. of 1 N NaOH and allow to stand to dissolve (may require gentle heat). Dilute with sterile water (less 2 ml.) to desired concentration.

*Courtesy of John A. Washington II, Head, Section of Clinical Microbiology, Mayo Clinic.

†Example: 1 mg. = 825 µg. ("activity standard"/µg.). 50 mg. = 50 × 825 = 41,250 µg. Therefore, add 41.2 ml. of diluent to give 1,000 µg./ml.

ceptions: microaerophilic streptococci, which are frequently isolated from cases of subacute bacterial endocarditis, strictly anaerobic streptococci, *Bacteroides* species, and clostridia should be tested in fluid thioglycollate medium enriched with hemin and vitamin K.; some strains may require additional enrichments to support good growth. In the case of all fastidious organisms the growth requirements should be determined before sensitivity tests are carried out, in order that the fluid medium supporting the most luxuriant and rapid growth may be selected for the procedure. Hemoglobinophilic organisms, such as *Haemophilus* species, must necessarily be tested in Mueller-Hinton broth containing 1% rabbit blood. The blood may be added to the broth before it is distributed into the test tubes, or it may be added with the inoculum.

Procedure for preparing serial dilutions and determining susceptibility

1. Thaw the frozen stock solution of the antibiotic(s) required and dilute 1:5 with sterile Mueller-Hinton broth. This gives a **working solution** containing 200 μ g. or units each. For bacitracin a concentration of 100 units per ml. may be used.
2. Select 10 clear, sterile, cotton-

plugged or capped test tubes of small size (13 \times 100 mm.) and mark from 1 to 10.

3. Using aseptic technique, pipette 0.5 ml. of dilution broth into tubes 2 through 10. Do this for each antibiotic to be tested.
4. Add 0.5 ml. of the working solution (200 μ g./ml.) of the antibiotic into tubes 1 and 2. Mix contents of the second tube well and transfer 0.5 ml. to tube 3. Mix well and transfer 0.5 ml. to tube 4, continuing this procedure to tube 9. Discard 0.5 ml. from tube 9; the tenth tube receives no antibiotic and serves as the control. Use a **separate** pipet for each transfer to avoid any carry-over.
5. To all tubes add 0.5 ml. of an inoculum containing approximately 10^5 to 10^6 organisms per milliliter. This may be prepared in most instances by making a 1:1,000 dilution in broth of an overnight (6-hour if a rapidly growing organism) broth culture of the organism to be tested. With slow-growing organisms, such as microaerophilic streptococci, *Bacteroides*, and so forth, it may be necessary to use cultures in thioglycollate medium up to 48 hours old. If numerous antibiotics are to be tested,

Table 34-2. Antibiotic serial dilution—tube setup

TUBE	DILUENT (MEDIUM) ADDED (ML.)	ANTIBIOTIC ADDED	DILUTED CULTURE ADDED (ML.)	FINAL ANTIBIOTIC CONCENTRATION	
				ALL BUT BACITRACIN (UNITS OR μ G.)	BACITRACIN (UNITS)
1	None	0.5 ml. working solution	0.5	100	50
2	0.5	0.5 ml. working solution	0.5	50	25
3	0.5	0.5 ml. from tube 2	0.5	25	12.5
4	0.5	0.5 ml. from tube 3	0.5	12.5	6.25
5	0.5	0.5 ml. from tube 4	0.5	6.25	3.125
6	0.5	0.5 ml. from tube 5	0.5	3.125	1.56
7	0.5	0.5 ml. from tube 6	0.5	1.56	0.78
8	0.5	0.5 ml. from tube 7	0.5	0.78	0.39
9	0.5	0.5 ml. from tube 8*	0.5	0.39	0.19
10	0.5	None	0.5	Zero	Zero

*Discard 0.5 ml. from tube 9.

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CONCENTRATION

BACITRACIN (UNITS)

50
25
12.5
6.25
3.125
1.56
0.78
0.39
0.19
Zero

prepare sufficient inoculum in a flask,
for uniformity.

The final volume in each tube is
now 1 ml., and the antibiotic range
covered in the series is from 100 μ g.
or units to 0.39 μ g. or unit per milli-
liter, in twofold steps (Table 34-2).

For organisms highly susceptible
to antibiotics, such as streptococci
and pneumococci, a lower range of
antibiotic dilutions may be employed
by further diluting the working solu-
tion (200 μ g. or units per milliliter)
1:10. Then, by the serial twofold di-
lution technique shown in Table
34-2, the final concentrations will be
10, 5, 2.5, 1.25, 0.63, 0.3, 0.15, 0.08
and 0.04 μ g. or units per milliliter.

6. Incubate the series at 36° C. and ex-
amine macroscopically for **evidence
of growth**. Incubate the tubes only as
long as it is necessary for the control
tube to show turbid growth; usually
12 to 18 hours is the optimal time.
The last tube, that is, the lowest con-
centration of the antibiotic in the
series showing no growth, is taken
as the MIC of the antibiotic and is
expressed as micrograms (or units)
per milliliter. It is well to remember
that in the serial dilution technique
there is a possible error equivalent to
one tube dilution, so that the MIC
values are not necessarily actual val-
ues but are near true values.

To determine the MBC, pipette 0.5
ml. of each tube of the tube dilution
set that shows no visible turbidity
into 12 ml. of infusion agar, mix, and
make a pour plate. Having obtained
a colony count of the **initial** inocu-
lum by making a pour plate of the
1:1,000 dilution when setting up the
test, one may then calculate the
lowest concentration of the antimi-
crobial that provided a 99.9% and
100% bactericidal activity, by com-
paring colony counts, after an appro-
priate incubation period of 48 to 72
hours.

AGAR PLATE DILUTION METHOD FOR DETERMINING SUSCEPTIBILITY TO ANTIBIOTICS

The agar plate dilution method¹ is simi-
lar in principle to the tube dilution meth-
od, except that a solid medium is used.
Mueller-Hinton agar is recommended and
is prepared in 100-ml. amounts. Some
workers incorporate 5% blood or heated
blood in the medium when using it for or-
ganisms that require enriched media, such
as pneumococci, streptococci, and *Haemo-*
philus. There appears to be no significant
inactivation of the antibiotics by the addi-
tion of the blood.

Procedure for preparing serial dilutions

Prepare twofold serial dilutions* of the
stock antibiotics, as described in the pre-
vious section, using at least ten times the
volumes indicated. Stock solutions con-
taining 1,000 μ g. per milliliter are most
useful, since decimal dilutions are readily
prepared from these. For example, to
prepare a 10 μ g. per milliliter plate, add 1
ml. of the stock to 100 ml. of melted and
cooled agar and pour plates of the same.

Preparation of plate dilutions

Melt and cool sufficient screw-capped
flasks of agar medium for the number of
plates to be prepared (about 20 ml. of me-
dium is required per 90 mm. diameter
plate) and allow to equilibrate in a water
bath at 50° before adding the antibiotic.
Add the required amount of the various
antibiotic dilutions to each flask, mix gen-
tly by inversion, and pour into plates.†
Allow the agar to harden and store in the
refrigerator at 5° C. until used, preferably
within 24 hours (and not after 1 week) of
preparation.‡

*Some workers prefer final dilutions to contain 20,
10, 5, 1, 0.1, and 0.01 μ g. per milliliter of medium.

†It is not recommended that the antibiotic dilutions
and culture medium be mixed directly in the plates;
this may produce uneven distribution of the antibiot-
ic in the agar.

‡Media containing unstable antibiotics, such as am-
picillin, should be prepared twice weekly.

Inoculation of plates

The inoculum size should be adjusted to contain approximately 10^8 organisms per milliliter (equivalent to a McFarland standard of 1 or 2); this will ensure dense, nearly confluent growth on a control plate containing no antibiotic.

Spot inoculation of the plates is made with a 1-mm. loop (approximately 0.001 ml.), a capillary pipet, or, preferably, by using the inocula replicator of Steers and co-workers.¹⁷ In this device, each single manipulation will release thirty-six different cultures from the prongs on a replicator head to the surface of a 100×15 mm. square plastic plate (Falcon) containing agar to a depth of 3.0 mm. Each prong will deliver about 0.001 ml.; thirty-six inoculations can thus be made simultaneously.

In using the Steers replicating device, it is recommended that one space on each plate be allocated to a marking solution (for proper orientation of the plate), one space for testing the viability of the test strain, and two spaces for controls—strains of gram-positive cocci and gram-negative bacilli of known stable MIC's to the antibiotics used. Thus, 32 spaces will then be available per plate for the testing of clinical isolates.

Organisms having a spreading tendency, such as *Proteus* and *Pseudomonas*, may be contained by the use of glass cylinders,* as suggested by Washington.²¹

Incubation and reading of tests

Incubate the plates at 36° C. for 16 to 18 hours and examine for the presence of growth. The lowest concentration of the antibiotic producing **complete inhibition of growth**† is taken as the end point. Partial inhibition can be observed readily by noting the gradual decrease in amount of growth until complete inhibition is ob-

tained. The control cultures on antibiotic-free media should show confluent growth.

STANDARDIZED DISC-AGAR DIFFUSION METHOD FOR DETERMINING SUSCEPTIBILITY TO ANTIBIOTICS

Perhaps the most useful, and certainly the most used, laboratory test for antibiotic susceptibility is the antibiotic disc-agar diffusion procedure, usually called the **disc method**. Its simplicity, speed of performance, economy, and reproducibility (under standardized conditions) makes it ideally suitable for the busy diagnostic laboratory when the more laborious dilution methods previously described may not be practiced.

In this method, as originally described by Bondi and associates,⁴ filter paper discs that have been impregnated with various antimicrobial agents of specific concentrations are carefully placed on an agar culture plate that has been inoculated with a culture of the bacterium to be tested. The plate is incubated overnight and observed the following morning for a **zone of growth inhibition** around the disc containing the agent to which the organism is **susceptible**, whereas a **resistant** organism will grow up to (and under) the periphery of the disc.

No attempt will be made here to discuss the complex physicochemical reactions that take place during the diffusion of the antibiotic into the agar gel or the dynamics of bacterial growth on the substrate—the reader is referred to publications by Ericson^{6,7} for details concerning these.

In recent years numerous attempts have been made to standardize the disc procedure, including the work of Bauer, Kirby, and co-workers,³ Ericsson,⁷ the World Health Organization (WHO), the Food and Drug Administration,⁹ and most recently, the National Committee for Clinical Laboratory Standards (NCCLS). Because the revised *Tentative Standards*¹⁴ recommended by the Subcommittee on Antimicrobial Susceptibility Testing of the NCCLS appears to be the most explicit in

*12 × 12 mm. Raschig rings, Scientific Glass Apparatus Co., Bloomfield, N. J.

†A very fine growth or a few visible colonies also may occur when the Steers replicator is used; this may be disregarded in the reading of the test.

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AGAR DIFFUSION TESTING ANTIBIOTICS

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methodology, these recommendations are the ones that will be presented herein.

Numerous proficiency testing surveys, including a recent nationwide laboratory evaluation by the Center for Disease Control (CDC)¹¹ of the disc procedure have revealed that (1) the procedure as practiced is not standardized and (2) there are numerous variables that may contribute to these discrepancies. Among those which have been identified are the following:

1. Selection and concentration of antimicrobial discs
2. Selection, volume, and age of plating medium
3. Storage and handling of discs
4. Methodology of testing
5. Criteria used for interpreting results

Selection of antimicrobial discs

The following basic set of drugs* and their concentrations are recommended for routine susceptibility testing:

Ampicillin	10 µg.
Bacitracin	10 U.
Carbenicillin ¹	100 µg.
Cephaloridine	30 µg.
Cephalothin	30 µg.
Chloramphenicol	30 µg.
Clindamycin	2 µg.
Colistin (Polymyxin E)	10 µg.
Doxycycline	30 µg.
Erythromycin	15 µg.
Gentamicin	10 µg.
Kanamycin	30 µg.
Lincomycin	2 µg.
Methicillin	5 µg.
Nafcillin and oxacillin	1 µg.
Nalidixic acid	30 µg.
Neomycin	30 µg.
Nitrofurantoin	300 µg.
Penicillin G	10 U.
Polymyxin B	300 U.
Streptomycin	10 µg.
Sulfonamides	300 µg.
Tetracycline	30 µg.
Vancomycin	30 µg.

A basic set of discs for routine testing against the commonly isolated microorganisms is listed on p. 322.

*Available from Baltimore Biological Laboratory, Cockeysville, Md.; Difco Laboratories, Detroit; Pfizer Diagnostics, Flushing, N. Y.; and others.

Selection of plating medium

Although an ideal medium has not yet been perfected for the disc test, the NCCLS Subcommittee considers Mueller-Hinton agar the best compromise for routine susceptibility testing, since it shows good batch-to-batch uniformity and is low in tetracycline and sulfonamide inhibitors. With the addition of 5% defibrinated sheep, horse, or other animal blood, it will support the growth of the more fastidious pathogens that will not grow on the non-enriched medium. When required, the blood-containing medium may be "chocolatized," for testing *Haemophilus* species.

Mueller-Hinton agar* is prepared according to the manufacturer's directions and should be immediately cooled in a 50° C. water bath after removal from the autoclave. This is then poured into sterile dishes (on a level, horizontal surface) to a uniform depth of 4 mm.; this is equivalent to approximately 60 ml. in a 140-mm. (internal diameter) plate, or approximately 25 ml. for 90-mm. plates. After cooling at room temperature, the plates may be used the same day, or stored in the refrigerator at 2° to 8° C. for not more than 7 days, unless some method of minimizing water loss from evaporation is taken.† As a sterility control, several plates from each batch of blood-containing Mueller-Hinton agar should be incubated at 36° C. for 24 hours or longer but not used subsequently.

Each batch of Mueller-Hinton agar should be checked for pH when prepared; it should be pH 7.2 to 7.4 at room temperature. This may be tested by macerating a small amount of the medium in a little distilled water, or by allowing a little of the medium in a small beaker to gel around the pH meter electrode,‡ and reading the pH.

*Available from Baltimore Biological Laboratory, Cockeysville, Md.; Difco Laboratories, Detroit; and others.

†Such as wrapping in polystyrene plastic.

‡If available, a surface electrode is desirable.

Just before the medium is used, the plates should be placed in a 36° C. incubator with lids partly ajar, until excess surface moisture has evaporated—usually requiring 10 minutes.

Storage and handling of discs

Antibiotic susceptibility test discs are generally supplied in separate containers with a desiccant* and should be kept under refrigeration (below 10° C.). Discs containing the penicillins (including ampicillin and carbenicillin) and cephalosporin drugs should always be kept frozen (at less than -14° C.) to maintain their potency; a small working supply may be refrigerated for up to 1 week. For long-term storage, discs are best kept in the frozen state until needed.

As they are required, the unopened containers are removed from the refrigerator or freezer 1 or 2 hours before the discs are to be used and allowed to adjust to room temperature, in order to minimize condensation resulting from warm air reaching the cold containers. If disc dispensers are utilized, they should be equipped with tight covers and supplied with a satisfactory desiccant; when not in use, they should also be refrigerated.

Manufacturer's expiration dates should be noted and listed; discs must be discarded on their expiration date.

Preparation of inoculum

It has been shown by various workers that when certified antibiotic discs and a single standard culture medium are used, the greatest factor contributing to reproducibility of the disc test is the control of the inoculum size.

The currently recommended method of preparing a standardized inoculum is as follows:

1. With a wire loop, the tops of four or five isolated colonies of a similar morphological type are transferred to

a tube containing 4 to 5 ml. of soybean-casein digest broth.*

2. The broth is incubated at 36° C. until its turbidity exceeds that of the standard (described in step 3). This usually requires 2 to 5 hours' incubation.
3. The turbidity is then adjusted to a barium sulfate standard that is prepared by adding 0.5 ml. of 1.175% w/v barium chloride hydrate ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) to 99.5 ml. of 1% v/v (0.36N) sulfuric acid. The standard is distributed in screw-capped tubes of the same size as the ones used in the broth culture, approximately 4 to 6 ml. per tube, which are then tightly sealed and stored at room temperature in the dark. Fresh standards must be prepared at least once every 6 months, although a recent publication suggests that the solution remains stable for a much longer period when heat-sealed and stored in the dark.²²
4. The barium sulfate standard must be vigorously agitated in a Vortex shaker just before use, and the turbidity of the broth culture is then adjusted visually by adding sterile saline or broth, using an adequate light and comparing the tubes against a white background with a contrasting black line.

Inoculation of the test plates

Within 15 minutes of adjusting the density of the inoculum, a sterile cotton swab on a wooden applicator stick (plastic sticks are not satisfactory) is dipped into the standardized bacterial suspension and the excess fluid is removed by pressing against the inside of the tube above the fluid level. The swab is then used to streak the dried surface of a Mueller-Hinton plate in several planes (by rotating the

*Humidity, particularly high humidity, heat, and contamination are important deteriorating factors.¹⁰

*Trypticase soy broth, Baltimore Biological Laboratory, Cockeysville, Md.; tryptic soy broth, Difco Laboratories, Detroit; and others.

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justing the den- rible cotton swab ck (plastic sticks ipped into the ension and the l by pressing tube above the n used to streak Mueller-Hinton y rotating the

Biological Laborato- broth, Difco Lab-

plate approximately 60° each time) to ensure an even distribution of the inoculum.

Allow the inoculated plates to remain on a flat and level surface undisturbed for 3 to 5 minutes to allow for adsorption of excess moisture, then apply the discs, as described in the following section.

Placement of discs

With alcohol-flamed, fine-pointed forceps (cooled before using) or a disc dispenser,* the selected discs are placed on the inoculated plate and pressed firmly into the agar with a sterile forceps or needle, to ensure **complete contact** with the agar. The discs are distributed evenly in such a manner as to be no closer than 15 mm. from the edge of the petri dish and so that no two discs are closer than 24 mm. from center to center. Once a disc has been placed, it should not be moved, since some diffusion of the antibiotic occurs almost instantaneously.

An alternative method, using an agar overlay, has been described by Barry and colleagues.² This method is useful only for rapidly-growing organisms such as *Staphylococcus aureus*, the enteric bacilli, and *Pseudomonas aeruginosa*, and must be standardized to correspond with results obtained by the cotton swab-streak method already described.

The inoculated and disced plates are inverted and placed in the 36° C. incubator within 15 minutes after application of the discs. Incubation under increased CO₂ tension should not be practiced, since the interpretative zone sizes were developed under aerobic conditions; furthermore, CO₂ incubation may significantly alter the zone sizes.

Table 34-3 is presented as a practical guide in the selection of discs for routine susceptibility testing of facultative organisms isolated in clinical practice. Although

not identical to that recommended by the NCCLS,¹⁴ it has proved of value to clinicians at the Wilmington Medical Center, and has served as an aid in reducing the misuse or overuse of antibiotic agents in a large medical complex.

Reading of results

After incubation the relative susceptibility of the organism to the antibiotic is demonstrated by a clear zone of growth inhibition around the disc. This is the result of two processes: (1) diffusion of the antibiotic and (2) growth of the bacteria. As the antibiotic diffuses through the agar medium from the edge of the disc, its concentration progressively diminishes to a point where it is no longer inhibitory for the organism, which then grows freely. The size of this area of suppressed growth, the **zone of inhibition**, is determined by the concentration of the antibiotic present in the area. Therefore, within the limitations of the test, the **diameter of the inhibition zone** denotes the **relative susceptibility** to a particular antibiotic.

After 16 to 18 hours' incubation,* each plate is examined and the diameters of the complete inhibition zones are noted and measured, using reflected light and sliding calipers, a ruler, or a template prepared for this purpose and held on the bottom of the plate. The **end point**, measured to the nearest millimeter, should be taken as the area showing no visible growth that could be detected with the unaided eye. Faint growth or tiny colonies near the edge of the inhibition zones are ignored, as is the veil of swarming occurring in the inhibition zones of some strains of *Proteus* species. With sulfonamides, slight growth (with 80% or more of inhibition) is disregarded, and the margin of heavy growth is measured to determine the zone diameter.

Large colonies growing in an inhibition

*Dispensers for both the 90-mm. and 140-mm. Petri plates are available from Baltimore Biological Laboratory, Cockeysville, Md.; Difco Laboratories, Detroit; Pfizer Diagnostics, Flushing, N. Y.; and others.

*Microbial growth should be almost or just confluent; if only isolated colonies are present, the inoculum was too light and the test must be repeated.

Table 34-3. Schema for recommended antimicrobial discs*

ORGANISM	AM 10 µg.	CB 50 µg.	CF 30 µg.	CM 30 µg.	CC 2 µg.	EM 15 µg.	GM 10 µg.	KM 30 µg.	LN 2 µg.	DP 5 µg.	NA 30 µg.	NF 300 µg.	PN 10 u.	PB 300 u.	SM 10 µg.	TE 30 µg.
Gram-negative rods																
<i>Escherichia coli</i>	x	[x]	x	[x]			x	x			(x)	(x)		x	[x]	[x]
<i>Klebsiella-Enterobacter-Serratia</i>	x		x	[x]			x	x			(x)	(x)		x	[x]	[x]
Other enteric bacilli	x		x	[x]			x	x			(x)	(x)		x	[x]	[x]
<i>Proteus</i> species	x	[x]		[x]			x	x			(x)	(x)			[x]	[x]
<i>Pseudomonas aeruginosa</i> , <i>Ps. sp.</i>		x		[x]			x				(x)			x	[x]	[x]
Other nonfermentative bacilli	x	x		[x]			x	x			(x)	(x)		x	[x]	[x]
Gram-positive cocci																
<i>Staphylococcus aureus</i>			x	[x]	x	x	[x]		[x]	x			x			[x]
<i>Streptococcus pyogenes</i> (Group A)						[x]			[x]				[x]			[x]
Group D streptococci, including enterococci	x		x	[x]		x	[x]						x		[x]	[x]
<i>Streptococcus pneumoniae</i>			[x]													
Other streptococci (alpha, and so forth)	x		x	[x]					[x]				x			[x]
Miscellaneous groups						x							x			[x]
<i>Neisseria meningitidis</i>	x		x	[x]									x			[x]
<i>Haemophilus influenzae</i>	x		x	[x]									x		[x]	[x]
Other facultative organisms																

*From the Section of Microbiology and the Infectious Disease Research Laboratory, Wilmington, Medical Center, Wilmington, Del. Abbreviations: AM, ampicillin; CB, carbenicillin; CF, cephalothin; CM, chloramphenicol; CC, clindamycin; EM, erythromycin; GM, gentamicin; KM, kanamycin; LN, lincomycin; DP, methicillin; NA, nalidixic acid; NF, nitrofurantoin; PN, penicillin G; PB, polymyxin B; SM, streptomycin; TE, tetracycline. x, Recommended for use with the species indicated; (x), recommended for use with urine isolates only; [x], recom

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Table 34-4. Zone size interpretative chart, Kirby-Bauer method*

ANTIBIOTIC OR CHEMOTHERAPEUTIC AGENT	DISC POTENCY	INHIBITION ZONE DIAMETER TO NEAREST MM.		
		RESISTANT	INTERMEDIATE	SENSITIVE
Ampicillin ¹				
Enterobacteriaceae and enterococci	10 µg.	11 or less	12-13	14 or more
Staphylococci		20 or less	21-28	29 or more
<i>Haemophilus</i>		19 or less	—	20 or more
Bacitracin	10 U.	8 or less	9-12	13 or more
Carbenicillin	100 µg.			
<i>Pseudomonas</i> sp.		13 or less	14-16	17 or more
<i>Proteus</i> and <i>Escherichia coli</i>		17 or less	18-22	23 or more
Cephaloridine	30 µg.	14 or less	15-17	18 or more
Cephalothin	30 µg.	14 or less	15-17	18 or more
Chloramphenicol	30 µg.	12 or less	13-17	18 or more
Clindamycin ²	2 µg.	14 or less	15-16	17 or more
Colistin (polymyxin E) ³	10 µg.	8 or less	9-10	11 or more
Doxycycline	30 µg.	12 or less	13-15	16 or more
Erythromycin	15 µg.	13 or less	14-17	18 or more
Gentamicin	10 µg.	12 or less	—	13 or more
Kanamycin	30 µg.	13 or less	14-17	18 or more
Lincomycin	2 µg.	9 or less	10-14	15 or more
Methicillin ⁴	5 µg.	9 or less	10-13	14 or more
Nafcillin and oxacillin	1 µg.	10 or less	11-12	13 or more
Nalidixic acid ⁵	30 µg.	13 or less	14-18	19 or more
Neomycin	30 µg.	12 or less	13-16	17 or more
Nitrofurantoin ⁵	300 µg.	14 or less	15-16	17 or more
Penicillin G				
Staphylococci	10 U.	20 or less	21-28	29 or more
Other organisms ⁶	10 U.	11 or less	12-21 ⁶	22 or more
Polymyxin B ³	300 U.	8 or less	9-11	12 or more
Rifampin (when testing <i>Neisseria meningitis</i> susceptibility only)	5 µg.	24 or less	—	25 or more
Streptomycin	10 µg.	11 or less	12-14	15 or more
Sulfonamides ^{5,7}	300 µg.	12 or less	13-16	17 or more
Tetracycline ⁸	30 µg.	14 or less	15-18	19 or more
Trimethoprim / sulfamethoxazole	25 µg.	10 or less	11-15	16 or more
Vancomycin	30 µg.	9 or less	10-11	12 or more

*Courtesy Alfred W. Bauer, Group Medical Center, Seattle, Wash., and John C. Sherris and W. Lawrence Drew, University Hospital, Seattle, Wash. See also reference 3 at end of chapter. Updated and modified by other investigators. See references 9 and 14.

¹The ampicillin disc is used for testing susceptibility to both ampicillin and hetacillin.

²The clindamycin disc is used for testing susceptibility to both clindamycin and lincomycin.

³The polymyxins diffuse poorly in agar, and the accuracy of the diffusion method is less than with other antibiotics. Resistance is always significant, but some relatively resistant strains of *Klebsiella* and *Enterobacter* may give zones in the lower end of the sensitive range (up to 15 mm.). When treatment of systemic infections due to susceptible strains is considered, it is wise to confirm the results of a diffusion test with a dilution method.

⁴The methicillin disc is used for testing susceptibility to all penicillin-resistant penicillins: methicillin, cloxacillin, dicloxacillin, oxacillin, and nafcillin. Methicillin-resistant strains of *Staphylococcus aureus* are best detected at 30°C.

⁵Urinary tract infections only.

⁶This category includes some organisms, such as enterococci and gram-negative bacilli, that may cause systemic infections treatable by high doses of penicillin G.

⁷Any of the commercially available 300 or 250 µg. sulfonamide discs can be used with the same standards of zone interpretation.

⁸The tetracycline disc is used for testing susceptibility to all the tetracyclines: chlortetracycline, demeclocycline, doxycycline, methacycline, oxytetracycline, rolitetracycline, minocycline, and tetracycline.

Haemophilus influenzae	As indicated by identity of isolate				[x]
	x	[x]	x		[x]
Other facultative organisms					

*From the Section of Microbiology and the Infectious Disease Research Laboratory, Wilmington, Medical Center, Wilmington, Del. Abbreviations: AM, ampicillin; CB, carbenicillin; CF, cephalothin; CM, chloramphenicol; CC, clindamycin; EM, erythromycin; GM, gentamicin; KN, kanamycin; LN, lincomycin; DR, methicillin; NA, nalidixic acid; NF, nitrofurantoin; PN, penicillin G; PB, polymyxin B; SM, streptomycin; TE, tetracycline. x, Recommended for use with the species indicated; (x), recommended for use with urine isolates only; [x], recommended for use with blood culture isolates only.

zone may actually be a different bacterial species (a mixed, rather than a pure, culture) and should be subcultured, reidentified, and retested.

Interpretation of zone sizes

The diameters of the inhibition zones are then interpreted by referring to Table 34-4, which represents the NCCLS subcommittee's present recommendations.

The term "susceptible" implies that an infection caused by the strain tested may be expected to respond favorably to the indicated antimicrobial for that type of infection and pathogen. "Resistant" strains, on the other hand, are not inhibited completely by therapeutic concentrations. "Intermediate" implies that the isolant may respond to unusually high concentrations of the agent, due either to high dosage levels or in areas, such as the urinary tract, where the drug is concentrated. In other circumstances, intermediate results might warrant further testing if alternative agents are not available.

Limitations of the test

This modified Bauer-Kirby procedure has been standardized for testing rapidly growing isolants, particularly members of the Enterobacteriaceae, *Staph. aureus*, and *Pseudomonas* species; limited experience also suggests that the interpretative standards hold for *Haemophilus* and streptococci, if blood ("chocolatized" if required) is added to the Mueller-Hinton agar. *Streptococcus pyogenes* and *S. pneumoniae* are generally susceptible to penicillin G and are not routinely tested; however, in those patients hypersensitive to penicillin, the isolant may be tested against erythromycin or lincomycin.

In general, fastidious organisms requiring an increased CO₂ tension or an anaerobic atmosphere, or whose growth rate is unusually slow, do not lend themselves to susceptibility testing by the standardized disc-agar diffusion method; agar plate or broth dilution test procedures are recommended. Likewise, testing of *Neisseria*

gonorrhoeae by the described procedure is not recommended. Because of the interest recently generated by early reports on reliable susceptibility testing of anaerobes using the disc procedure, a subsequent section will consider this technique.

Quality control procedures

It is essential that some form of quality control be carried out in performing the disc procedure, to ensure precision and accuracy of the test results. The NCCLS subcommittee recommends that the tests be monitored daily with stock cultures of the Seattle strains of *Staph. aureus* (American Type Culture Collection 25923) and *Escherichia coli* (ATCC 25922), using antibiotic discs representative of those to be used in the testing of clinical isolants.¹⁴ These cultures may be grown on soy-casein digest agar slants and stored under refrigeration (4° to 8° C.), and should be subcultured to fresh slants every 2 weeks.

For testing, the cultures are inoculated to soy-casein digest broth tubes, which are incubated overnight and streaked to agar plates to obtain isolated colonies; these are then picked to broth and tested as described in the preceding sections.

The control strains may be used as long as there is no significant change in the mean inhibition zone diameters not otherwise attributable to technical error. If such changes occur, fresh strains should be obtained from a reference laboratory or other reliable source. Individual values of zone diameters and their permissible differences are indicated in Table 34-5.

Table 34-5 represents a more precise computation, based on standard statistical methods, than previous publications. It is described in NCCLS's *Revised Tentative Standards* (May 1973), free copies of which may be obtained from the Committee.*

Recently, it has been recommended that a well-characterized and confirmed strain

*National Committee for Clinical Laboratory Standards, Los Angeles, Calif.

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Table 34-5. Maximum acceptable standard deviations and mean zone diameters that should be expected with the *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923)*

ANTIMICROBIC	DISC CONTENT	MAXIMUM ACCEPTABLE STAND. DEV.	CURRENTLY ACCEPTED TRUE MEAN ZONE DIAMETER (MM)	
			<i>E. COLI</i>	<i>S. AUREUS</i>
Penicillin	10 units	2.9	†	31.5
Ampicillin	10 µg.			
Staphylococci		2.9	†	29.5
Enteric bacilli and enterococci		1.3	17.5	†
Methicillin	5 µg.	1.6	†	19.5
Nafcillin				
and oxacillin	1 µg.	1.3	†	—
Cephalothin	30 µg.	1.3	20.5	31.0
Cephalexin	30 µg.	1.6	—	†
Carbenicillin	50 µg.			
<i>Pseudomonas</i> sp.		1.3	—	†
<i>Proteus</i> and <i>E. coli</i>		1.6	—	†
Chloramphenicol	30 µg.	1.9	24.0	22.5
Tetracycline	30 µg.	1.6	21.5	23.5
Erythromycin	15 µg.	1.6	11.0	26.0
Lincomycin	2 µg.	1.9	†	—
Clindamycin	2 µg.	1.6	†	—
Kanamycin	30 µg.	1.6	21.0	22.5
Neomycin	30 µg.	1.6	20.0	22.0
Streptomycin	10 µg.	1.3	16.0	18.0
Gentamicin	10 µg.	1.3	22.5	23.0
Sulfonamides	300 µg.	1.6	—	†
Nitrofurantoin	300 µg.	1.6	—	†
Nalidixic acid	30 µg.	1.9	—	†
Polymyxin B	300 units	1.3	14.0	†
Vancomycin	30 µg.	1.3	†	17.0

*NCCLS Subcommittee on Antimicrobial Susceptibility Testing: Performance standards for antimicrobial disc susceptibility tests as used in clinical laboratories, revised tentative standards, May 1973. In Balows, A., editor: Current techniques for antibiotic susceptibility testing, Springfield, Ill., 1974, Charles C Thomas, Publisher.

†Data not relevant; — data not yet established.

of *Pseudomonas aeruginosa* be added to the quality control system.¹¹ Apparently, some lots of Mueller-Hinton agar may contain increased concentrations of Ca⁺⁺ and Mg⁺⁺. Since *Staph. aureus* and *E. coli* are not effected by these ions, they will demonstrate no changes in zone sizes, but growth of *Ps. aeruginosa* is enhanced and will therefore demonstrate smaller zones. Thus, the effect of increased concentrations of these cations would influence the interpretation of susceptibility and should be predetermined.

Susceptibility testing of anaerobes

With the diverse spectrum of activity of various antimicrobial agents against clinically significant anaerobes, it is clearly apparent that a simple, rapid, reliable test for their susceptibility is in demand. Attempts to adapt the Bauer-Kirby disc-agar diffusion technique for predicting sensitivity of anaerobes has been found to be generally unsatisfactory,¹⁹ but a number of workers in the field of anaerobic bacteriology are attempting to correlate the zone diameters obtained by the disc test, with the MIC's

obtained by either broth or agar dilution techniques, with varying degrees of success. Chief among these has been the work of Sutter and colleagues,¹⁸ who have obtained a statistically good correlation with most of the antibiotics tested against a variety of known strains of anaerobes. The authors point out, however, that if predictions of the antibiotic sensitivity of **unidentified** isolants are to be made by the disc-diffusion procedure, it may be necessary to establish separate criteria for organisms that have different growth rates—slow, moderately rapid, or rapid—since this is one of the major variables that affect zone sizes.

Therefore, we feel that until a standardized, reproducible, and clinically correlated disc-diffusion technique for predicting antibiotic susceptibility of significant anaerobes becomes available, the reader is best directed to the employment of methodologies utilizing broth or agar dilution procedures.^{13,14}

As a guide to the microbiologist and clinician, Table 34-6 from a recent publication by Finegold and co-workers* is presented. This is based on their correlation of in vitro laboratory findings with an evaluation of clinical effectiveness.

Other uses for antibiotic discs

One unexpected advantage of performing disc-sensitivity tests on primary plates inoculated with clinical specimens likely to contain more than a single pathogenic species (such as sputum, throat swabs, or urine) is the likelihood of **uncovering** organisms overgrown by other bacterial species. For example, an agar plate inoculated with a mixed culture of staphylococci and streptococci and "disced" with two different antibiotics may result in two distinct patterns of inhibition zones. One zone may show an inhibition of the staphylococci, whereas colonies of the streptococci may be growing within that zone.

*In Kagan, B. M., editor: Antimicrobial therapy, ed. 2, Philadelphia, 1973, W. B. Saunders Co.

Table 34-6. Susceptibility of anaerobes to antimicrobial agents*

ANTIMICROBIAL AGENT	MICROAEROPHILIC AND ANAEROBIC COCCI	BACTEROIDES FRAGILIS	BACTEROIDES MELANINOGENICUS	FUSO-BACTERIUM VARIIUM	OTHER FUSOBACTERIUM SPECIES	EUBACTERIUM AND ACTINOMYCES	CLOSTRIDIUM PERFRINGENS	OTHER CLOSTRIDIA
Penicillin G	++++	+	+++	+++†	+++	+++	+++†	+++
Lincomycin	+++	++	+++	++	+++	++	++	+
Clindamycin	+++	+++	+++	+++	+++	+++	+++	+++
Metronidazole	++	+++	+++	+++	+++	?	+++	+++
Chloramphenicol	+++	+++	+++	+++	+++	+++	+++	+++
Tetracycline	++	++	+++	+++	+++	++	++	++
Erythromycin	++ to +++	+	++	++	++ to +++	++	++	++ to +++
Vancomycin	++ to +++	+	+	+	+	++ to +++	++	++ to +++

*Chart courtesy of Sydney M. Finegold. From Kagan, B. M., editor: Antimicrobial therapy, ed. 2, Philadelphia, 1973, W. B. Saunders Co. ++++, Drug of choice; +++ good, activity; ++, moderate activity; +, poor or inconsistent activity.

†A few strains are resistant.

‡Rare strains resistant.

§Based on old studies (resistance may have developed subsequently).

- ed from the patient. Also prepare a pour plate using 1 ml. of the inoculum.
5. Incubate the test at 36° C. for 18 to 24 hours and examine. The bacteriostatic end point is taken as the highest dilution in which no visible growth occurs. Because of the inherent turbidity of some sera, it is recommended that subcultures be made from each tube to a sector of a blood agar plate. To determine bactericidal end points, transfer 0.05 ml. from each tube showing no growth to a tube of thioglycollate medium. Mix and incubate at 36° C. for 72 hours. The tube showing no growth in thioglycollate is taken as the end point. Good growth should be evident in the control tube.
 6. In cases where the organism grows slowly, a loopful of an overnight broth culture may be used as the inoculum. When microaerophilic or anaerobic bacteria have been isolated, the tubes should be incubated anaerobically.
 7. Since thioglycollate medium contains sufficient agar to permit the growth of discrete colonies, one can determine by inspection the number of colonies growing out, and thus the degree of killing. It is thus recommended that one report the results as follows:
 - a. Complete killing at serum dilution____, with no growth in subculture.
 - b. Partial inhibition at serum dilution____, with 1 to 2+ growth in subculture.
 - c. Record and report the presence of less than 10 colonies at the serum dilution observed.
 8. If the percentage of bactericidal activity is required, the following may be carried out:
 - a. Pipette 0.5 ml. of each tube showing no gross turbidity into tubes containing 12 ml. of melted and cooled (45° to 50° C.) brain-heart infusion agar, mix, and make pour plates.
 - b. Incubate for 72 hours at 36° C.
 - c. On the basis of the inoculum colony count (step 4), calculate the lowest titers of serum dilution showing 99.9% and 100% bactericidal activity, and report accordingly.
 9. Schlichter indicated that optimal antibiotic dosage (either single or combined drugs) had been achieved when a bactericidal level of 1:2 (complete inhibition in the first two tubes) had been demonstrated; others, however, believe that a bactericidal level of 1:8 should be the minimum effective level in problem cases. Dosage is adjusted according to results of the test and is maintained for the duration of the infection.

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Burkholderia cepacia and Cystic Fibrosis: Do Natural Environments Present a Potential Hazard?

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An environmental survey of 55 sites yielded only 12 *Burkholderia cepacia* isolates, none of which displayed the phenotypic properties of a multiresistant epidemic strain associated with pulmonary colonization in patients with cystic fibrosis. Although the environment probably poses a low risk for patients with cystic fibrosis as a source of *B. cepacia*, the pathogenic potential of individual environmental strains remains unclear. We advise caution in the development of *B. cepacia* as a biocontrol agent.

First described in 1950 by Burkholder (5) as the cause of soft rot of onions, *Burkholderia cepacia* (basonym *Pseudomonas cepacia*) is now of wider and increasing interest in agriculture, biotechnology, and medicine. Reasons for this interest include the organism's abilities to promote plant growth by antagonizing soilborne plant pathogens (15, 16, 25), to degrade hydrocarbons and thus assist in the bioremediation of contaminated soil and water (11), and to cause opportunistic human infections, particularly in patients with chronic granulomatous disease (22, 30) and cystic fibrosis (CF) (1, 14, 19, 36). In patients with CF, *B. cepacia* is now a major pathogen because of the following: first, its association with cepacia syndrome, a rapidly fatal necrotizing pneumonia, sometimes accompanied by septicemia (19); second, its innate multiresistance to antibiotics (23, 34); third, person-to-person transmission through social contact (14, 24, 38); and fourth, the risk of nosocomial acquisition (40). In the last decade, there have been increased attempts to reduce the risk of *B. cepacia* transmission by segregation of *B. cepacia*-colonized patients and publication of guidelines concerned with hygiene and social behavior (1, 29, 42). The social effects of segregation on patients with CF and organizations for people with CF are psychologically devastating, and the policy has not been universally welcomed, particularly in centers for patients with CF in which *B. cepacia* colonization or transmission has remained low (1, 33, 39). The introduction of hygiene guidelines and segregation has undoubtedly reduced acquisition of *B. cepacia* in patients with CF (14, 38, 42), but acquisition has not been eliminated. The source of *B. cepacia* responsible for new acquisitions is unclear, and there is increasing concern about the risk that the environment may present as a source of this opportunistic saprophyte (10, 20). At present, rational judgments on this issue are frustrated by the following uncertainties: first, the distribution of *B. cepacia* in natural environments; second, the pathogenic potential of environmental isolates; and third, the potential hazards associated with the development and use of *B. cepacia* as a biological control agent.

B. cepacia has been cultured from a range of environmental sources, including soil, water, and vegetation (2, 15, 26), and from clinical material (17). There are few reports, however, of prospective studies of the organism's natural habitats, and there is little documented evidence to support the use of the

term ubiquitous to describe the organism's environmental distribution. LiPuma et al. (24) cited previous failures to recover *B. cepacia* from environmental surfaces to support person-to-person transmission as the primary mode of *B. cepacia* acquisition in patients with CF. In a subsequent study, *B. cepacia* was cultured from only 1% of samples obtained from homes and 4.5% of samples obtained from salad bars and food stores (10). The use of the term environmental isolates also can be misleading since in epidemiological studies of nosocomial *B. cepacia* infections, it is not always possible to identify whether isolates cultured from environmental surfaces have contaminated patients or vice versa. The aims of our study were to investigate prospectively the distribution of *B. cepacia* in other natural environments and to obtain authentic environmental isolates for future comparative studies with clinical isolates to investigate *B. cepacia* virulence factors and to determine the pathogenic potential of environmental strains.

A large botanical complex was chosen for this survey to provide an extensive range of soils, aquatic sites, and vegetation located in a natural temperate climate and tropical, subtropical, and arid microclimates within greenhouses. Fifty-five samples of soil, soil rhizosphere, water, and vegetation were collected and cultured for *B. cepacia* by using selective media and screening procedures adapted from two previous surveillance studies (10, 28). Prior to sampling, swabs were first moistened in a selective broth that consisted of Malka minimal broth (35) supplemented with polymyxin (300 U/ml) and plated directly onto selective agar that consisted of 300 U of polymyxin per ml and 100 µg of ticarcillin per ml (Mast cepacia agar; Mast Diagnostics, Ltd., Bootle, United Kingdom). Water samples (100 ml) were filtered (pore size, 0.2 µm; Millipore). The same swabs, filter membranes, soils, and vegetation were transferred to Malka minimal broth supplemented with polymyxin, incubated at 30°C for 5 days, and subcultured to the selective agar described. After 48 h at 30°C, bacterial colonies were subcultured to MacConkey agar and fluorescent pigment-enhancing King's B medium (21). After a further 48 h at 30°C, nonfluorescent, non-lactose fermenters were screened on arginine-glucose medium, *B. cepacia* isolates were presumptively identified by biochemical reactions (13), and identification was confirmed by the API 20 NE system (bioMérieux, Basingstoke, United Kingdom). Individual *B. cepacia* isolates were then investigated for catalase production (32), lipopolysaccharide (LPS) content by polyacrylamide gel electrophoresis following proteinase K extraction as previously described (6), and antimicrobial susceptibility by an agar dilution method on Mueller-Hinton agar (23), with sensitivity defined in terms of the British

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TABLE 1. Antibiotic resistance of 48 clinical and 25 environmental isolates of *B. cepacia* with sensitivity defined in terms of breakpoints

Antibiotic	Breakpoint (mg/liter) ^a	No. of resistant isolates (%)	
		Clinical	Environmental ^b
Piperacillin	64	20 (42)	1 (4)
Ceftazidime	2	39 (81)	4 (16)
Meropenem	4	25 (52)	2 (8)
Chloramphenicol	8	44 (92)	18 (72)
Ciprofloxacin	4	18 (38)	2 (8)
PD 127391	4	10 (21)	0 (0)
PD 131628	4	14 (29)	0 (0)

^a Defined by the British Society for Antimicrobial Chemotherapy Working Party (4), apart from PD 127391 and PD 131628, which have been defined by Levin et al. (23).

^b Includes 10 isolates in this study and 15 isolates previously held in our collection.

Society for Antimicrobial Chemotherapy Working Party breakpoints (4) (Table 1). Pulsed-field gel electrophoresis (PFGE) was performed by the technique of Vasil et al. with some modifications (43). Bacteria were grown in nutrient broth no. 2 (Oxoid, Basingstoke, United Kingdom) that contained 0.5% yeast extract (Difco, Surrey, United Kingdom). Cells were washed with 75 mM NaCl–25 mM EDTA, standardized to an optical density at 590 nm of 1.0, and mixed with an equal volume of molten 1% low-melting-point preparative-grade agarose (Bio-Rad, Hemel Hempstead, United Kingdom). DNA restricted with *Xba*I (TCTAGA) and *Spe*I (ACTAGT) (Gibco BRL, Paisley, United Kingdom) was separated by PFGE by using the CHEF DRII system (Bio-Rad) with pulses of 2.9 to 35.4 s at 200 V for 22 h at 14°C. Gels were stained with ethidium bromide at a concentration of 1 µg/ml for 15 min and then destained in distilled water for 30 min.

B. cepacia was cultured from 12 (21.8%) of 55 samples and sites (Table 2). This recovery rate exceeds that reported by Fisher and colleagues (10) for homes (1%) and food stores (4.5%); however, both studies indicate that it is an exaggeration to describe *B. cepacia* as ubiquitous or widely distributed in nature. The data also emphasize the biological puzzle posed by Palleroni (31), namely, if *B. cepacia* is so nutritionally versatile, why is this species not more readily cultured from natural habitats? In our study, *B. cepacia* isolates were not recovered

from healthy or diseased cacti or from soil within the cactus greenhouse; the majority of recovery sites were in moist soil or soil rhizosphere, which suggests that these environments are natural habitats for this microbial saprophyte.

Previous studies (6,14) have shown that *B. cepacia* J2315, an epidemic strain associated with morbidity and mortality in patients with CF and epidemic outbreaks in regional clinics for those with CF in the United Kingdom, has unusual phenotypic characteristics, including a dry rough colonial morphotype, dark melanin-like pigmentation, a sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile indicative of rough LPS, and multiresistance to co-trimoxazole and all of the antibiotics listed in Table 1. These characteristics were not observed for any of the 12 environmental isolates of *B. cepacia*. The results of antibiotic sensitivity testing are shown in Table 1; in general, environmental isolates, including 15 previously held in our collection, showed greater sensitivity than did CF isolates of *B. cepacia*. Interestingly, however, epidemic strain J2315 and 10 of 12 environmental isolates were catalase positive, a characteristic of opportunistic pathogens responsible for recurrent life-threatening infections in patients with chronic granulomatous disease (22).

Genomic analysis of DNA from environmental isolates of *B. cepacia* by PFGE following endonuclease digestion with *Spe*I and *Xba*I illustrated the genomic diversity of this species. With one exception, each of these 12 isolates produced a distinct endonuclease restriction profile which also differed from the PFGE profiles of epidemic strain J2315 and 10 other clinical isolates of *B. cepacia* cultured from patients with CF.

Accumulated evidence from this study and previous reports (10, 18) suggests that compared with the heavily colonized respiratory secretions of *B. cepacia*-colonized patients with CF, the environment is a relatively low-risk source of *B. cepacia* for patients with CF. Honicky et al. recovered *B. cepacia* from only 4 (3 from lake water and 1 from ice water from a picnic jug) of 58 environmental samples in a study of *B. cepacia* acquisition in patients with CF who attended summer camps (18). Furthermore, genomic fingerprinting of *B. cepacia* cultures from the 16 (6.6%) of 244 campers whose acquisition of *B. cepacia* was associated with camp attendance showed that the majority of new colonizations involved strains shared by other camp attenders and that no patient with CF had acquired a strain which shared a genotype found in environmental isolates.

An important caveat to the conclusion that the environment is not a major source of *B. cepacia* infection in humans concerns the development and release of *B. cepacia* as a biological agent to control plant disease and soil decontamination. In a recent report, Bevivino et al. (3) concluded that environmental isolates probably pose little threat in human disease since they lack the abilities to produce gelatinase and a hydroxamate siderophore and to adhere to human uroepithelial cells. This conclusion needs to be treated with considerable caution. Only two environmental isolates and two clinical isolates were investigated; furthermore, there is no evidence that the putative virulence determinants investigated in this study play a role in human infections, including colonization and pathogenesis in patients with CF (27).

Equally relevant to the issue of the use of *B. cepacia* in biological control are the questions of whether clinical and environmental *B. cepacia* organisms represent two distinct groups and, in particular, whether environmental strains cause human infections, either de novo or through adaptation. In respect to the latter, in longitudinal studies of *B. cepacia*-colonized patients with CF, there is no phenotypic evidence of adaptation in vivo, which is so characteristic of mucoid alginate-producing *Pseudomonas aeruginosa* (27). Previous re-

TABLE 2. Environmental sites in botanical gardens from which *B. cepacia* strains were cultured

Isolate	Site	Area
J2534	Rotting bark	Orchid and cycad house
J2535	Rotting bark	Orchid and cycad house
J2536	Soil	Gardens
J2537	Soil	Rhododendron house
J2538	Soil	Rhododendron house
J2539 ^a	Rhizosphere of <i>Hohenbergia stalata</i>	Temperate aquatic house
J2540	Rhizosphere of banana plant	Tropical aquatic house
J2541	Rhizosphere of <i>Epidendrum o'brienianum</i>	Orchid and cycad house
J2542	Rhizosphere of <i>Nautilocalyx lynchii</i>	Orchid and cycad house
J2543 ^a	Pond water	Tropical aquatic house
J2552	Rhizosphere of <i>Carludoucas palmata</i>	Tropical aquatic house
J2553	Sansevieria leaf	Tropical palm house

^a Identical PFGE profiles.

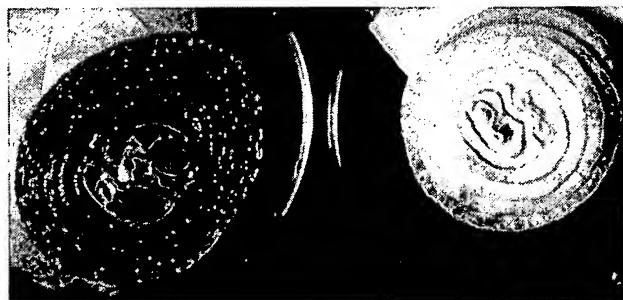


FIG. 1. Soft rot of onion segments inoculated with *B. cepacia* clinical isolate J2315 (left) and an uninoculated, undamaged control (right), both incubated at 30°C for 72 h.

ports (12, 44) concluded that the two groups of *B. cepacia* are distinct and that clinical isolates do not have the ability to act as phytopathogens; however, this distinction is unfounded since most clinical isolates, including epidemic strain J2315, readily cause soft rot of onions (Fig. 1). In turn, evidence that environmental isolates have the ability to cause human infection is provided by the macerated, hyperkeratotic foot lesions encountered by troops during swamp training, known as swamp rot (41). The distinctive phenotypic properties of multiresistant epidemic *B. cepacia* isolates, including J2315 (14, 37), suggest that a subpopulation of *B. cepacia* may have a predilection for the lungs of patients with CF. Recent evidence (37) that such strains possess characteristics of *B. cepacia* and the closely related phytopathogen *Burkholderia gladioli* and that the genomic constitution of *B. cepacia* may facilitate genetic rearrangements (7) supports speculation that the transition of environmental *B. cepacia* isolates from phytopathogen to pulmonary colonization in patients with CF is enhanced by intraspecies adaptation or the emergence of bacterial hybrids of the two species.

At present, unequivocal statements on the potential of environmental *B. cepacia* isolates to cause infections in patients with CF and in other immunocompromised hosts are frustrated by our scanty knowledge of the virulence factors and mechanisms of pathogenesis involved (27). The recent development of mutant mice with CF (9) and the demonstration that *B. cepacia* J2315 causes pneumonia in mice with CF but not in control animals (8) provide a useful model to investigate the pathogenic potential of environmental *B. cepacia* isolates for patients with CF.

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Case Management — Continued

fore and after learning HIV-positive results indicate a decline in high-risk behavior, the findings in this report likely underestimate the behavior changes.

Transmission of HIV can be interrupted by assisting persons with HIV infection in reducing their unsafe sexual and drug-use behaviors. HIV-prevention case management is an early intervention strategy to provide this assistance through counseling, education, psychosocial referrals, and behavioral skills training (7). Since 1992, HIV prevention case management has been identified as a specific program priority for state and local health departments and community-based organizations (CBOs) receiving HIV-prevention funding from CDC (1). CDC directly funds 19 CBOs to provide HIV-prevention case management, and many health departments have implemented this HIV-prevention service.

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Epidemiologic Notes and Reports

***Pseudomonas cepacia*
at Summer Camps for Persons with Cystic Fibrosis**

Pseudomonas cepacia (PC) is a multidrug-resistant, gram-negative bacillus that causes chronic colonization and infection of the respiratory tract of persons with cystic fibrosis (CF). PC colonization is usually difficult to eradicate with antimicrobial therapy and, in some patients, infection is associated with rapid decline in pulmonary function, increased hospitalization, and earlier death (1-4). Previous studies have suggested person-to-person transmission of PC both within and outside of hospitals (2,3,5-7). However, possible transmission of PC at CF summer camps—sites for physical and psychosocial therapy for many patients—has not been well characterized. To assess the risk for PC transmission in this setting, in 1987 and 1990, the CF Foundation and CDC conducted epidemiologic investigations in four CF summer camps in Michigan, Ohio, Utah, and Ontario, Canada. This report summarizes the results of these studies.

Pseudomonas cepacia — Continued**Michigan**

In June 1987, 55 previously known PC-negative patients who were PC-culture negative immediately before attending camp attended a week-long CF camp with 36 other campers known to be colonized or infected with PC. The camp was staffed by 79 volunteer medical, paramedical, and laypersons who served as counselors and administered respiratory therapy and chest physiotherapy to campers. To determine the incidence of sputum conversion from PC-negative to PC-positive among campers, sputum or throat cultures were performed on all participants on arrival at, daily during, and within 3 months after camp.

To determine exposures of PC-negative campers to PC-positive patients or to particular camp staff and potential environmental sources of PC at camp, two investigators visually monitored campers' activities and administered a daily written questionnaire to each camper and/or the camper's counselor. None of the 55 CF campers with initially PC-negative sputum had PC-positive sputum cultures on departure from camp. However, five (9%) were PC-positive on their first follow-up culture within 2–13 weeks after camp. None were exposed to PC outside of the camp setting during this period. All five had reported close contact with PC-positive patients at camp, including participating in the same activities together for most of the day (four patients), hugging (three), lip-to-cheek kissing (one), and sharing toothpaste or finger food with (two) a PC-positive camper.

PC isolates from all five converters had the same ribotype (i.e., the restriction fragment-length polymorphism banding patterns were identical or had one- or two-band differences) as isolates from one or more PC-colonized campers and different from those of control isolates from other CF campers from other summer camps or CF centers. Of the five converters, three had PC with the same ribotype as that of isolates from PC-colonized campers with whom they had reported close contact.

Of 22 environmental cultures, three lake water samples grew PC. All three had an identical ribotype distinct from any of the PC isolates from campers.

Ohio, Utah, and Ontario

From June through August 1990, a study was conducted at three CF summer camps attended by PC-negative and PC-positive patients in Ohio, Utah, and Ontario. Sputum or throat cultures were performed on campers on their arrival at camp, every 7 days until the end of camp, and 14–30 days after camp. To compare the incidence of sputum conversion from PC-negative to PC-positive of CF patients at camp with that outside of camp, sputum cultures were also performed on consenting noncamper CF patients who were known to be PC-negative and who, during the 2 weeks before camp, visited outpatient clinics or were hospitalized at the CF centers that the campers attended. The noncampers' sputum cultures were repeated 14–30 days after their corresponding CF-center summer camp ended.

Overall, of 191 CF patients who were PC-negative on arrival at camp, 181 completed their after-camp follow-up. Their cumulative incidence of PC sputum conversion was 11 (6%) of 181. The CF campers' risk for acquiring PC was approximately 12 times (Woolf's estimate of relative risk [RR]=11.7, lower 95% confidence limit=1.7) that of 92 noncamper controls, none of whom acquired PC during the study period. The increased risk for acquiring PC was not associated with older age or more severe underlying CF—host factors that predispose CF patients to develop PC coloni-

Pseudomonas cepacia — Continued

zation. Compared with noncampers, PC-negative campers were younger and had milder CF. In addition, PC-negative campers and noncampers had similar sex distributions.

The risk for conversion to PC-positive was directly proportionate to the prevalence of PC-positive persons at camp: zero of 84 PC-negative campers in the Ohio camp (3% of attendees were PC-positive on entry); two (4%) of 47 PC-negative campers in the Utah camp (16% were positive on entry); and nine (18%) of 50 PC-negative campers in the Ontario camp (38% were PC-positive on entry) ($p < 0.001$, chi-square test for linear trends). The risk for conversion also was increased in the camp with the longer duration: nine (18%) of 50 in the Ontario camp (duration: 4 weeks), compared with two (2%) of 131 in Ohio and Utah combined (duration for each: 1 week) ($RR = 11.8$; 95% $CI = 2.7-53.5$).

Risk-factor assessment based on daily (in Ohio and Utah) or weekly (in Ontario) written questionnaires indicated that the risk for sputum conversion was higher in those who reported sharing an eating utensil ($RR = 8.9$; 95% $CI = 2.7-30.1$), dancing ($RR = 4.2$; 95% $CI = 1.2-15.4$), or sleeping in the same cabin with a PC-positive camper ($RR = 3.7$; 95% $CI = 1.1-12.3$).

Of 11 campers whose sputum converted from PC-negative to PC-positive, nine had PC isolates with the same ribotype as that of PC isolates of other campers at their respective camps, and two had a common ribotype distinct from those of PC isolates of 33 known PC-positive persons in the same camp. Of the nine campers whose PC ribotypes matched those of other campers, four reported any contact with the campers whose isolates were of the same ribotype as theirs, including sleeping in the same cabin with and/or spending more than 4 hours per day in the company of a PC-positive camper.

Of 36 environmental cultures, one (ice water obtained from a picnic jug at the Utah camp) grew PC; this PC isolate had a distinct ribotype that differed from any of the PC isolates from Utah campers.

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Editorial Note: Studies to determine the risk for and mechanisms of PC acquisition by CF patients have been limited by the unknown sensitivity of sputum or throat cultures in detecting PC in the CF patient's respiratory tract and, consequently, by the difficulty in determining when PC is acquired by the patient. Despite these limitations, the epidemiologic and laboratory findings in this report suggest that PC can be acquired at CF summer camps, and person-to-person transmission is a likely mode of spread. Factors that may have contributed to an increased risk for PC acquisition at the camps include a high prevalence of PC-positive CF campers (>5%) or prolonged (>1 week)

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Pseudomonas cepacia — Continued

duration of camp, probably reflecting increased opportunity for frequent, close, and prolonged contact between campers.

The degree to which contact-isolation precautions (i.e., handwashing, gloving, gowning, cohorting of CF patients by their PC-colonization status, and discarding contaminated articles) (8) were followed at each camp in this report was not assessed; therefore, the impact of recommended precautions for preventing nosocomially acquired PC at camp is unknown. In CF camps where transmission of PC has been suspected or where the prevalence of PC-positive campers exceeds 5%, and/or camp duration is longer than 1 week, camp personnel should either fully implement contact-isolation precautions at the camp or prohibit PC-positive and PC-negative CF patients from attending camp together (8). In areas with a high prevalence of PC-positive patients, separate CF summer camps for PC-positive and PC-negative patients may be feasible.

These recommendations are dependent on adequate procedures for screening patients before camp. Therefore, sputum or throat cultures should be appropriately collected from patients with CF and transported to and processed in a laboratory that routinely uses PC-selective media and by personnel who are proficient in isolating and identifying PC from sputum of patients with CF (9).

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*Current Trends***Mortality Trends and Leading Causes of Death
Among Adolescents and Young Adults — United States, 1979-1988**

Approximately three fourths of the more than 40,000 deaths each year among persons aged 10-24 years in the United States are related to preventable causes such as motor-vehicle crashes (37%), homicide (14%), suicide (12%), and other injuries (e.g., drowning, poisoning, and burns) (12%). To characterize changes in leading causes of

Pseudomonas cepacia: Biology, mechanisms of virulence, epidemiology

Pseudomonas cepacia, originally described as a plant pathogen, has emerged as an important cause of infection in altered hosts, particularly in the hospital setting. This organism's ability to survive and proliferate in a variety of solutions, medications, and even disinfectants and antiseptics has resulted in numerous clusters of common-source nosocomial infections. Many patients exposed to *P. cepacia* are merely colonized, but serious infections, including surgical and burn wound infections, bacteremia, meningitis, pneumonia, peritonitis, and urinary tract infections, are not rare. The virulence properties of this pathogen remain poorly characterized. Recently, *P. cepacia* has been reported in some cystic fibrosis centers as an increasingly frequent pulmonary pathogen. This trend has caused considerable concern because of reports of occasional cases of fulminant necrotizing pneumonia and bacteremia. Conversely, many patients with CF who become colonized with this organism have no ill effects. The epidemiology of *P. cepacia* in the CF population is unclear, but some patients probably acquire the organism from colonized siblings with CF. Circumstantial evidence suggests that the organism may also be acquired in the hospital. Treatment of infections is exceedingly difficult, particularly in patients with CF, because *P. cepacia* is resistant to a broad range of antibiotics. (J PEDIATR 1986;108(2):806-812)

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Pseudomonas cepacia, first described as a cause of soft rot in onions in 1950,¹ is now recognized as an important opportunistic pathogen in hospitalized patients and other compromised hosts. Although many patients are colonized with *P. cepacia* without noticeable ill effects, others experience severe, life-threatening infections. Recent reports from a number of centers²⁻⁷ indicate that patients with cystic fibrosis are at particularly high risk for colonization and infection with *P. cepacia*. Indeed, the CF population is the principal group affected by *P. cepacia* at the present time. The difficulty in treating *P. cepacia* pulmonary infection in these patients has stimulated increasing interest in the microbiology, epidemiology, and virulence of this organism.

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P. cepacia has had a variety of names in the past, including *Pseudomonas kingii*, *Pseudomonas multivorans*, and EO-1 (eugonic oxidizers group 1), but the identity of all of these organisms has been clearly established.^{8,9} On the basis of nucleic acid homologies, *P. cepacia* has been found to be closely related to *Pseudomonas mallei* and *P. pseudomallei* (the agents of glanders and melioidosis, respectively), *P. pickettii* (a rare cause of

CF Cystic fibrosis

human infections), and several pseudomonal plant pathogens.^{10,11} It is only distantly related to the much more common and well-known opportunist, *Pseudomonas aeruginosa*. Like other members of its genus, *P. cepacia* is ubiquitous in the environment, where it is frequently associated with soil, water, and plants. It is an incredibly versatile organism, capable of utilizing a wide variety of nutrients for growth. Suitable substrates range from sim-

ple salts such as ammonium acetate¹² to complex organic molecules such as 2,4,5-trichlorophenoxyacetic acid, the principal herbicide in "agent orange."¹³ Even penicillin can serve as the sole source of carbon, nitrogen, and energy.¹⁴ This metabolic versatility has been exploited by genetic engineering of *P. cepacia* strains that utilize environmental chemical contaminants.

P. cepacia survives in a variety of adverse conditions (Table I). It thrives in natural water sources^{15, 16} and can proliferate in tap or distilled water,^{17, 18} presumably by utilizing trace elements and low concentrations of organic materials. This characteristic, coupled with its resistance to a variety of commonly used disinfectants, antiseptics, and preservatives, makes *P. cepacia* a formidable nosocomial pathogen.

Numerous outbreaks of *P. cepacia* colonization and infection have been caused by contaminated solutions, pharmaceuticals, and medical devices.¹⁹ Contaminated dilute aqueous quaternary ammonium disinfectant has caused epidemics of infection, ultimately leading the Centers for Disease Control to recommend against its use in hospitals.¹⁸⁻²⁰ In one instance, *P. cepacia* persisted for 14 years in an inorganic salts solution "preserved" with 0.05% benzalkonium chloride.¹² By gradually increasing the concentration of benzalkonium chloride, strains were easily derived that grew in 16% disinfectant. *P. cepacia* can also persist in aqueous chlorhexidine^{21, 22} and povidone iodine.²³

P. cepacia is virtually nonpathogenic in normal, healthy individuals. Even direct injection of contaminated fluids into immunocompetent patients may result in only transient fever or colonization.^{19, 22} However, when patients have severely altered host defenses (as in the case of burn victims or patients with CF) or have indwelling catheters or other medical devices, serious infections may occur. Reported infections include endocarditis (particularly in intravenous drug abusers),²⁴ bacteremia,²⁵ postoperative and burn wound infections,²⁶ peritonitis,²⁷ osteomyelitis and septic arthritis,²⁸ meningitis,²⁹ and lung abscess and pneumonia.³⁰⁻³² The infections complicating chronic granulomatous disease³³⁻³⁵ are particularly interesting because they tend to occur in individuals who have had no prior evidence of a host defense problem.

The association of *P. cepacia* with pulmonary disease in CF was first noted during a study of aminoglycoside resistance in pseudomonads isolated from sputum cultures in this population.² Of the 200 patients studied from 1974 to 1978, eight were found to be colonized with *P. cepacia*. More recently, some cystic fibrosis centers have reported a dramatic increase in the rate of isolation of *P. cepacia* from sputum. For example, at the Rainbow Babies and Childrens Hospital in Cleveland the annual incidence of

Table I. Sources of isolates of *Pseudomonas cepacia* (selected)

Decayed onions	Ultrasonic nebulizers
Soil	Infant incubators
Rotting tree trunks	Respirators
Tap water	Humidifiers
Distilled water	Pressure transducers
Saline solution	Intravenous fluids
Raw and pasteurized milk	Normal serum albumin
Topical anesthetics	Cryoprecipitate
Aerosol antibiotics	Dialysis machines
Methylprednisolone	Disinfectants
Urinary catheter kits	Baby lotions

Adapted from Gilardi GL. Lab Management 1983;21:29-32.

pulmonary colonization with *P. cepacia* increased from 3.3% to 8.2% from 1979 to 1983, and the prevalence of patients with *P. cepacia* in their sputum increased from 5.1% to 20.0%.³⁶ An increase in the prevalence of *P. cepacia* colonization also was reported by the CF group at the Toronto Hospital for Sick Children during a similar period.^{5, 6} Despite these reports, the magnitude of the *P. cepacia* problem in CF remains undefined. It is possible that the apparent increase in the prevalence of *P. cepacia* in some centers is, at least in part, an artifact of increased laboratory awareness, and this may be exacerbated now that efficient selective culture media are available.^{37, 38} Most centers have noted only sporadic isolates of *P. cepacia*. In other centers, such as St. Christopher's Hospital for Children in Philadelphia, the incidence of *P. cepacia* isolations has not changed dramatically, and the prevalence of colonization has gradually declined (Dr. D. Schidlow, personal communication).

The appearance of *P. cepacia* in the CF population has caused considerable alarm, not only because of the increasing number of colonized patients being recognized at some centers but also because some investigators have reported that colonization tends to be associated with accelerated clinical deterioration. Thomassen et al.,³⁹ in Cleveland, noted that patients colonized with *P. cepacia* seemed to have more serious lung disease and a poorer prognosis than patients colonized only with *P. aeruginosa*. Isles et al.,⁶ in Toronto, found that some patients, particularly girls and young women, who acquired *P. cepacia* experienced a rapid decline in pulmonary function.⁶ A small subset of patients who had been clinically stable with relatively mild lung disease experienced an acute, fulminant course characterized by high fever, elevated sedimentation rate and white blood cell count, progressive pulmonary failure, and death. Pathologic examination of the lung revealed severe necrotizing pneumonia. From 1977 to 1981, isolation of *P. cepacia* from patients who died increased from 17% to 42%. Similarly, four young adult women in Cleveland had

relentless deterioration, leukocytosis, spiking fevers, and death within several weeks to a few months after colonization with *P. cepacia*.⁴⁰ *P. cepacia* was recovered from blood cultures in all four patients. *P. cepacia* bacteremia in CF patients is striking; *P. aeruginosa*, the organism that colonizes the vast majority of patients with CF, virtually never causes bacteremia.^{3,41}

Although these reports are of great concern, many patients colonized with *P. cepacia* have no detectable change in their clinical course,^{39,42} and certain patients colonized with *P. aeruginosa* experience unpredictable, sudden pulmonary deterioration. Moreover, regardless of whether the lung is colonized with *P. cepacia* or *P. aeruginosa*, CF pulmonary disease is ultimately lethal. Carefully controlled epidemiologic studies in a large CF population will be required to establish the true virulence of *P. cepacia*. A recently completed study at St. Christopher's Hospital for Children, performed in collaboration with the Centers for Disease Control, represents an important first step.⁷ This case-control study suggested that patients colonized with *P. cepacia* tend to be hospitalized longer and die sooner than comparison subjects matched for severity of illness. However, the matching criteria used in this study (mild, moderate, and advanced disease categorized by Schwachman score⁴³) are broad and subjective, and it may be difficult for any single center to define a sufficient number of controls matched by more precise indicators of the severity of underlying pulmonary disease.

It is not clear how patients with CF initially become colonized with *P. cepacia*. Patients probably encounter the organism frequently in the community, and colonization may be favored by the selective pressure of the prolonged oral antibiotic therapy. Accumulating evidence suggests that many patients acquire *P. cepacia* either in the hospital or through contact with colonized siblings. The clustering of *P. cepacia* in a few centers implies that acquisition of the organism may be associated with specific hospital exposures or practices. Moreover, colonization appears to be correlated with recent or concurrent hospitalization^{7,36,39} or a recent visit to a hospital clinic (Dr. D. Schidlow, personal communication). In studies not involving CF patients, contaminated topical anesthetics⁴⁴ and solutions used in bronchoscopy, aerosolized antibiotics,⁴⁵ and contaminated nebulizers^{30,46} all have been associated with *P. cepacia* pulmonary colonization and infection. Inadequate disinfection of inhalation therapy equipment was an important cause of gram-negative pulmonary infection until the danger was recognized in the last decade.⁴⁷ Therefore, investigators in several centers performed extensive culture surveys to detect *P. cepacia* contamination of the

hospital environment. To date there has been no convincing evidence for an environmental reservoir in CF centers.

Attempts to demonstrate person-to-person spread of *P. cepacia* have yielded tantalizing clues, but the extent of transmissibility among CF patients is unclear. The risk of *P. cepacia* colonization is significantly increased if a sibling harbors the organism.^{7,36,39} Analysis by biotyping, serotyping, bacteriocin typing, and antibiograms suggests that some sibling pairs are colonized by the same strain.⁴⁸ Aside from this apparent sibling-to-sibling spread, direct evidence that *P. cepacia* is transmitted from person to person, either in the hospital or in other settings where CF patients congregate, has been difficult to obtain. Nevertheless, the possibility that *P. cepacia* might be acquired by contact with colonized patients has produced considerable anxiety in the CF community. In Cleveland, the CF group has responded by instituting a variety of procedures to reduce the risk of transmission, including physical separation of colonized and noncolonized patients in the hospital, reeducation of the staff concerning basic infection control procedures, and institution of separate summer camps. These measures have been associated with a sharp decline in the incidence of *P. cepacia* colonization, from 8.2% in 1983 to 1.7% in 1984.³⁶ Further studies are needed to confirm the efficacy of these precautions, which significantly alter the care routine and social life of CF patients.

Antimicrobial therapy for *P. cepacia* infections poses a significant challenge, because this pathogen is routinely resistant to many agents. In fact, resistance to the polymyxins and aminoglycoside antibiotics is useful in distinguishing this organism from other pseudomonads in the laboratory and in designing selective culture media.^{37,38,49} *P. cepacia* is also resistant to first- and second-generation cephalosporins and traditional antipseudomonal penicillins, such as ticarcillin. This is not surprising, because an inducible β -lactamase has been demonstrated in *P. cepacia*, which hydrolyzes many cephalosporins and penicillins.⁵⁰ Until recently, the most effective antibiotics available for the treatment of *P. cepacia* infections were trimethoprim-sulfamethoxazole and chloramphenicol^{19,44,51,52}; these antibiotics may still be useful in certain clinical settings. Treatment of CF poses a special problem; few strains remain susceptible to TMP-SMZ or chloramphenicol, and the aminoglycoside and penicillin antibiotics used to treat *P. aeruginosa* infections will increase the selective pressure favoring colonization with *P. cepacia*. Further studies are required to determine how the growing popularity of aerosolized antibiotics, such as polymyxin, carbenicillin, and tobramycin, in CF centers may affect

Table II. Serotyping of *Pseudomonas cepacia*

Isolate source	Serotype* (%)							Multi-type	Non-typable	Reference
	I	Ia	Ib	II	III	IV				
CF patients (n = 112)	50.9	2.7	0.9	0	1.8	3.6		33.8	6.3	Klinger et al. ⁶⁵
Non-CF patients (n = 65)	7.6	6.2	3.1	0	10.8	7.7		33.8	30.8	Klinger et al. ⁶⁵
Environmental (n = 34)	8.8	8.8	0	0	2.9	0		8.8	70.7	Klinger et al. ⁶⁵
Non-CF patients (n = 137)	21.4	31.7	30.3	8.3	8.3	†		‡	‡	Jonsson ⁶⁴

Serotyping scheme of Jonsson⁶⁴; serotype IV described in Klinger et al.⁶⁵

*Percentage of total strains (per source group) for indicated serotype.

†Serotype IV not described at time of study.⁶⁴

‡Polyagglutinating (multitype) and nontypable strains not indicated.⁶⁴

colonization with *P. cepacia*.^{45, 53} In addition, aerosol equipment might become contaminated with *P. cepacia*, resulting in aerosolization of the organism into the lung.^{47, 54}

Recently developed antibiotics, including several third-generation cephalosporins, thienamycin (imipenem), aztreonam, and ciprofloxacin, demonstrate some in vitro activity against *P. cepacia*.⁵⁵⁻⁵⁹ Of these agents, ceftazidime was believed to be particularly promising because most strains from patients with CF are susceptible in vitro.⁵⁸ Initial clinical experience with ceftazidime in the treatment of patients with *P. cepacia* infection, however, has been variable. Gold et al.,⁶⁰ administered 18 courses of ceftazidime to 14 CF patients with severe chronic lung disease. Clinical improvement occurred with six treatment courses. Eight treatment courses were classified as failures, and four patients died after six, seven, eight, and 29 days of therapy, respectively. These therapeutic failures could not be attributed to the development of resistance to ceftazidime because resistance was found in only one patient.⁶⁰ Subsequently, the same group treated a less severely ill population of colonized patients.⁴² Most patients improved clinically, as generally can be expected when CF patients are hospitalized and given antibiotics intravenously.⁶¹ Ceftazidime significantly decreased *P. aeruginosa* in the sputum, but there was no reduction in the density of *P. cepacia*. Kerckmar et al.⁶² and Blumer et al.⁶³ also noted clinical improvement after ceftazidime administration in the majority of patients who had failed to respond to conventional therapy.

Inasmuch as none of the antibiotics currently under development is likely to have a major impact on *P. cepacia* pulmonary infection in patients with CF, future research will focus on ways to prevent acquisition. Standard infection control techniques will eliminate the usual hospital reservoirs of *P. cepacia*. Barrier precautions may prove useful in limiting person-to-person transmission, should this prove to be a frequent occurrence. The development of

stable, reliable microbiologic typing systems would greatly facilitate study of the epidemiology of *P. cepacia* and permit a more rational approach to preventing further spread of the organism in the population with cystic fibrosis. Several typing systems have been proposed, but all must be considered preliminary. The first system to be described was based on 139 human isolates of EO-1 from the collection of the Centers for Disease Control.⁶⁴ Five serotypes (I, Ia, Ib, II, and III) were described, with types Ia and Ib most frequently encountered. Recent studies have defined an additional serotype, IV.⁶⁵ When recent isolates from CF patients, other patients, and environmental sources were serotyped, it was discovered that a large proportion of the CF isolates were type I, compared with only 8% of strains from other sources (Table II). Many strains (particularly from environmental sources) agglutinated in multiple antisera or were nontypable, limiting the usefulness of this system in its present form and suggesting the existence of additional serotypes.

Two other serotyping systems have been described in the past few years and have been used in limited epidemiologic studies. A French group⁶⁶ serotyped 285 isolates using a combination of seven O (somatic) and five H (flagellar) antisera. Strains from nosocomial outbreaks of *P. cepacia* in medical and surgical units in the Strasbourg area tended to have the same serotypes. For example, strains from a cluster of infection caused by contaminated bronchoscopes were serotype O5H5. Another system of 10 serogroups has been described by Japanese investigators.⁶⁷ With this system all but 12% of 105 strains could be serotyped.

Two biotyping systems have been proposed for *P. cepacia*.^{68, 69} These systems are appealing because each relies on a small number of biochemical reactions that could be performed easily by most laboratories. With these systems *P. cepacia* can be separated into four^{68, 69} and eight⁶⁹ biovars, respectively. *P. cepacia* can also be charac-

terized by bacteriocin production and sensitivity.⁷⁰ Very recently, Govan et al.⁷¹ reported that 95% of 400 strains were typable, falling into 44 cepacian types. Strains from documented outbreaks tended to have the same bacteriocin production and sensitivity patterns.

Very little is known about the virulence properties of *P. cepacia*. In the initial report by Burkholder¹ and in several subsequent studies,⁷² 50% to 79% of *P. cepacia* strains have had protease (gelatinase) activity. McKevitt and Woods⁷³ screened 48 *P. cepacia* isolates from patients with CF and found that the majority were proteolytic. Most strains also produced lipase; no exotoxin A- or exotoxin S-like activities were detected. At least one metalloproteinase of *P. cepacia* has been partially characterized.⁷⁴ Studies of proteolytic activity in *P. cepacia* are of particular interest, and proteases are believed to be important in the pathogenesis of *P. aeruginosa* pulmonary infections. Purified proteases from *P. aeruginosa* produce significant pulmonary damage when directly instilled into the lungs of experimental animals. In addition, recent work suggests that proteases of *P. aeruginosa* are the primary virulence factors in acute experimental pneumonia in guinea pigs.⁷⁵

Some strains of *P. cepacia* are capable of synthesizing phenazine pigments similar to those of *P. aeruginosa*.⁷⁶ The conditions for optimal pigment synthesis and the frequency of this trait among clinical strains are not known, but it is interesting to speculate on the extent to which these pigments may inhibit lymphocyte proliferation, as is seen with *P. aeruginosa*.⁷⁷

Despite the in vitro demonstration of these virulence properties, *P. cepacia* has little virulence in animal models. Using a guinea pig model of *Pseudomonas* pneumonia in which intratracheal inoculation of 10⁷ colony-forming units of *P. aeruginosa* reliably produces acute severe, fatal necrotizing pneumonia,⁷⁸ greater inocula of *P. cepacia* were required to produce even mild disease. An isolate from a patient who died of necrotizing *P. cepacia* pneumonia was no more virulent in this model than a strain from a colonized patient (J. Pennington, personal communication). In preliminary experiments (data not shown), we have seen persistence of *P. cepacia* for at least 2 weeks in a rat model of chronic pulmonary infection.⁷⁹ Pathologic changes were similar to those observed using *P. aeruginosa*, and no animals died (data not shown). *P. cepacia* is considerably less virulent than *P. aeruginosa* in the burned-mouse model, whether the organisms are injected intraperitoneally or inoculated onto the burned skin.⁸⁰ However, a highly proteolytic isolate from a patient with CF was found in cultures of the liver 5 days after inoculation of the burn wound, suggesting that bacteremia had occurred. A recent report by Brauner et al.²⁶ may provide a clinical corollary to this observation. Two cases

of *P. cepacia* bacteremia were noted subsequent to burn wound colonization, and strains from both patients were gelatinolytic.

Considerably more work will be needed to determine how *P. cepacia* produces infections in humans. Because *P. cepacia* infections can no longer be considered rare and are very difficult to treat, especially in patients with CF, there is an urgent need for further research.

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REVIEW ARTICLE

Burkholderia cepacia: medical, taxonomic and ecological issues

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The increasing challenge posed by multiresistant saprophytes in medical microbiology is strikingly demonstrated by the emergence of *Burkholderia* (formerly *Pseudomonas*) *cepacia* as an opportunist pathogen in immunocompromised patients, particularly individuals with chronic granulomatous disease and cystic fibrosis (CF). Best known previously as a phytopathogen and the cause of soft rot of onions, *B. cepacia* presents three major problems for the CF community: innate multiresistance to antimicrobial agents; person-to-person transmission of epidemic strains through nosocomial or social contacts; and 'cepacia syndrome', a fulminating fatal pneumonia, sometimes associated with septicaemia, that occurs in approximately 20% of colonised patients, including those with previously mild disease. Accumulated evidence to dispel earlier suggestions that the organism is avirulent and merely a marker of existing lung disease includes: case-controlled studies in CF patients; reports of serious infections in non-CF patients; in-vitro and in-vivo evidence that *B. cepacia* induces production of pro-inflammatory markers, including the major cytokine TNF α ; and histopathological evidence that exposure of transgenic CF mice to *B. cepacia* results in pneumonia. By the early 1990s, the use of selective culture media and DNA-based bacterial fingerprinting confirmed suspicions of epidemic person-to-person spread of *B. cepacia*. This evidence provided scientific justification for draconian and controversial measures for infection control, in particular, segregation of *B. cepacia*-colonised patients during treatment at CF centres and their exclusion from social gatherings and national conferences. Recently, molecular analyses of type strains and clinical isolates have revealed that isolates identified previously as *B. cepacia* belong to at least three distinct species and have increased concern regarding the reliability of current laboratory detection and identification systems. Clarification of the taxonomy of *B. cepacia*-like organisms and the pathogenic potential of environmental isolates remains a high priority, particularly when the organism's antifungal and degradative properties have created interest in its potential use as a biological control agent to improve crop yields and its use for the bioremediation of contaminated soils.

Introduction

'The development of multiresistance in major microbial pathogens is well-recognised; in contrast, little attention has been paid to the pathogenic potential of naturally resistant environmental saprophytes'.

Known originally as a phytopathogen, *Burkholderia cepacia* (previously *Pseudomonas cepacia*, *P. multi-*

vorans, *P. kingii*, 'Eugonic oxidiser 1') exhibits impressive nutritional versatility. Some microbes have an inherent or acquired ability to degrade antibiotics, but few have the ability to use penicillin as a sole carbon source [1] or to reduce onions to a macerated pulp! The earlier name, *P. multivorans*, reflected the organism's omnivorous appetite, but it was not until 1950 that its pathogenic potential was recognised when Burkholder identified the organism as the cause of soft rot of onions—particularly 'compromised' onions damaged during harvesting—and provided an appropriate species epithet (Latin: *cepia* = onion) [2]. In the early 1990s, following taxonomic re-appraisal, the RNA group II pseudomonads were recognised as

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the new genus *Burkholderia*, with *B. cepacia* as the type species [3]. At present, the genus *Burkholderia* comprises *B. cepacia*, *B. gladioli*, *B. mallei*, *B. pseudomallei*, *B. caryophylli*, and recently added to the group, *B. plantarii*, *B. glumae*, *B. vandii* [4], *B. cocovenenans* [5] and *B. vietnamiensis* [6].

The general characteristics of *B. cepacia* include the following: gram-negative, non-spore-forming, aerobic bacillus; motile with a respiratory metabolism and typically catalase- and oxidase-positive; various non-fluorescent pigments may be produced and poly- β -hydroxyalkanoates can be accumulated as reserve materials; the optimal temperature for growth is 30–35°C [7]. Recently, elegant molecular analyses have provided scientific evidence that may account for the organism's impressive versatility, including multilocus linkage disequilibrium analysis of environmental populations [8]—which suggested an extraordinarily high rate of recombination in *B. cepacia* relative to binary fission—and demonstration of multiple relicts and insertion sequences in type strains [9, 10].

The natural habitats of *B. cepacia* have been described as soil, water and vegetation [11]. However, it is a common but erroneous belief that *B. cepacia* is a ubiquitous saprophyte sharing similar environmental habitats with *Pseudomonas aeruginosa* and other pseudomonads. Extensive surveillance studies have shown that culture of *B. cepacia* from natural sources, including soil, water and plants, or from hospitals, foodstores, restaurant salad bars and patients' homes is surprisingly difficult, with detection rates of only 1–16% [12–16].

In agricultural microbiology, ecological awareness and an increasing incidence of pesticide-resistant pathogens have led to interest in *B. cepacia* as a potential agent for biological control and soil decontamination. *B. cepacia* produces several antimicrobial agents, including pyrrolnitrins, altericidins, cepalymins and bacteriocin-like agents [17–20], that inhibit bacterial and fungal phytopathogens and suppress tobacco wilt and other plant diseases [21]. *B. cepacia* is also capable of degrading industrial waste and herbicides, including 2,4,5-trichlorophenoxyacetic acid (2,4,5-T),

the principal ingredient of the highly potent 'agent orange' [22]. Indeed, *B. cepacia* has been shown to degrade 2,4,5-T in heavily contaminated soils at a rate up to 20 000-fold greater than other known degradative bacteria [23].

In contrast to its potential agricultural benefits, *B. cepacia* has also emerged as a multiresistant opportunist human pathogen, leading to concern about the relationship between environmental and clinical isolates [14, 24–26] and the potential hazards of releasing *B. cepacia* as a biological control agent [14, 24]. This review will provide an update on microbes currently described as *B. cepacia*, with particular focus on clinical, taxonomic and ecological issues (Table 1) associated with pulmonary infection in patients with cystic fibrosis (CF).

The emergence of *B. cepacia* as a human pathogen

Before the early 1980s, reports of human infections caused by *B. cepacia* were sporadic and generally restricted to hospitalised patients exposed to contaminated disinfectant and anaesthetic solutions in which this nutritionally adaptable saprophyte survives for long periods. Infections included those of soft tissues and the respiratory and urinary tracts, but bacteraemia also occurred, sometimes associated with endocarditis and septic shock [27–31]. A rising incidence of *B. cepacia* infection was noted during the early 1980s and, although in some cases culture of *B. cepacia* was thought to reflect mere colonisation or contamination rather than infection [11, 32], extensive analyses of USA databases of nosocomial infections confirmed a significant increase in clinically significant *B. cepacia* infections [33, 34]. The apparent propensity of *B. cepacia* to cause fatal pulmonary infections, as suggested by these analyses, is emphasised in patients with chronic granulomatous disease (CGD)—in whom *B. cepacia* pneumonia and septicaemia are life-threatening [35, 36]—and in its emergence as a major pathogen in patients with CF [37–39]. By the 1990s, disturbing reports also emerged of fatal cases of *B. cepacia* pneumonia and septicaemia in previously

Table 1. Major issues associated with *B. cepacia* and cystic fibrosis (CF)

- Is there convincing evidence to confirm that *B. cepacia* has pathogenic potential and is not merely a marker of pulmonary disease?
- Based on the success, but unpopularity, of segregation and advances in clarifying the taxonomy of the genus *Burkholderia*, should all *B. cepacia* be treated as equal? Can phenotypic or genomic markers be found which would identify highly transmissible or virulent clones?
- To what degree do natural environments represent a reservoir for *B. cepacia* and a hazard for CF patients? What hazards are associated with the development and use of *B. cepacia* as a biological control agent?
- Could an improved understanding of the host immune response, including enhanced cytokine induction by bacterial surface components, clarify the immunopathology of *B. cepacia* and lead to innovative forms of immunotherapy?
- At present, it is not possible to forecast the clinical outcome of *B. cepacia* colonisation. Can host and bacterial factors responsible for initial colonisation and poor clinical outcome be identified?
- Recently, it has been demonstrated that CF airway epithelia contain bactericidal activity that is inhibited reversibly by high NaCl concentrations. Does this killing potential include *B. cepacia* and is it host or strain specific?
- Ultimately, the identification of bacterial and host factors associated with transmission and virulence would assist greatly in the rational design of an effective *B. cepacia* vaccine.

healthy individuals [40, 41]. Community-acquired *B. cepacia* infections are uncommon, but the organism's pathogenic potential and the financial implications of antimicrobial therapy were recently strikingly demonstrated when an offshore oil worker developed multiple brain abscesses secondary to suppurative otitis media. Therapy involved four neurosurgical operations, an extensive period of hospitalisation and an antibiotic bill of £10K [42].

The above case also demonstrated an interesting and unexplained variability in antibiotic susceptibility profiles that has been observed in serial *B. cepacia* isolates from single patients and during epidemic outbreaks [43–46]. The mechanism responsible for variable susceptibility is unclear, but may be associated with the observation that migration of insertion sequences within the *B. cepacia* genome can affect the expression of genes that modulate antibiotic resistance [47].

B. cepacia and cystic fibrosis

During the last decade, the major clinical interest in *B. cepacia* has focused on its addition to the relatively narrow spectrum of microbial pathogens responsible for debilitating and ultimately fatal pulmonary infections in patients with CF [26, 39, 48, 49]. In the late 1980s, surveillance studies in the UK indicated a maximum prevalence of 7% [39, 50–52]; however, in some CF centres this later increased to approach the prevalence of 40% described in contemporary North American studies [53]. The three major issues concerning *B. cepacia* can be summarised as follows: 1, the clinical risk of rapid and fatal pulmonary decline, even in patients with previously mild disease; 2, patient-to-patient spread of epidemic strains within and between regional CF centres and between centres in the UK and North America; and 3, the innate multiresistance of most *B. cepacia* isolates to available antibiotics—which deprives patients of effective antimicrobial therapy [46, 54]—combined with the failure to reduce the bacterial population in sputum and a relatively poor clinical response even when the colonising strain exhibits in-vitro susceptibility.

The clinical significance of *B. cepacia* in CF patients was first described in 1984 in a seminal report by Isles *et al.* [37]. In addition to noting the increased prevalence of *B. cepacia* colonisation in patients attending Toronto clinics, Isles *et al.* described a rapid and unexpected clinical decline, including necrotising pneumonia and bacteraemia, that occurred in c. 20% of colonised patients. This acute clinical decline is sometimes referred to as 'cepacia syndrome' [37]. It is important to note that acute clinical deterioration and bacterial spread to sites other than the lung is not observed with the other major CF pathogens, *Staphylococcus aureus*, *Haemophilus influenzae* and *P. aeruginosa*.

The second major issue relating to *B. cepacia* arose in the mid 1980s as an increasing—but scientifically unproven—conviction held by some CF carers that the clustering of cases in some large North American clinics had arisen from cross-infection. At that time, an alternative explanation for clustering was the difficulty in culturing this relatively new pathogen from CF sputa [48]. As evidence, in a controlled study involving 115 North American CF centres, only 36 (31%) cultured the organism successfully from a seeded sputum specimen [55]. However, by the early 1990s, the availability of selective culture media [48] and awareness of the organism's cultural idiosyncrasies [56] indicated that regional variation in the prevalence of *B. cepacia* colonisation could not be explained simply by laboratory methodology. Furthermore, the development and use of bacterial fingerprinting techniques—including multilocus enzyme electrophoresis (MLEE), pyrolysis mass spectroscopy, PCR-ribotyping and pulsed-field gel electrophoresis (PFGE)—provided compelling evidence for person-to-person spread of *B. cepacia* through nosocomial and social contacts (Table 2) [25, 37, 46, 57–75] and, occasionally, in the absence of proven sputum colonisation [67]. Epidemiological data also provided scientific justification for the introduction of guidelines by national CF organisations to improve personal and hospital hygiene and, more controversially, for the implementation of segregation policies to limit contact between colonised and non-colonised individuals [76]. Surveillance studies show that segregation undoubtedly reduces the incidence of *B. cepacia* cross-infection [38, 62, 71, 77], but the strategy has not eliminated acquisition. Furthermore, the logistic and social consequences of draconian infection control measures reminiscent of mediaeval approaches to leprosy have not been accepted universally. In particular, the need for such measures has been questioned fiercely by patients and care-givers in CF centres where intensive surveillance has not revealed a high incidence or prevalence of *B. cepacia* colonisation.

A pathogen or a marker of lung disease?

In the 1970s, some microbiologists and clinicians considered *S. aureus* to be the only true microbial pathogen in CF patients and viewed *P. aeruginosa* as merely a marker of disease. A similar doubt has accompanied the emergence of *B. cepacia* and has exacerbated the controversy surrounding segregation of colonised individuals. In discussions of any potential opportunist pathogen, it is easy to find evidence of asymptomatic carriage; even *Salmonella typhi* and *Vibrio cholerae* do not invariably exhibit pathogenicity!

Clarification of the clinical relevance of *B. cepacia* is also thwarted by the fact that the available scientific evidence requires particularly careful analysis. There is an inclination to link bacterial transmissibility and virulence, and to categorise individual *B. cepacia*

Table 2. Evidence for and against person-to-person transmission of *B. cepacia*

Reference	Comments
A. Evidence in favour of person-to-person transmission	
Isles <i>et al.</i> [37]	Seminal paper: noted rising incidence of <i>B. cepacia</i> and cepacia syndrome in Canadian clinics
Thomassen <i>et al.</i> [57]	Fall in incidence after segregation
LiPuma <i>et al.</i> [58]	Prevalence of one ribotype in individual clinics
LiPuma <i>et al.</i> [59]	Ribotyping demonstrates person-to-person spread between two patients at a CF camp
Anderson <i>et al.</i> [60]	Nosocomial outbreak
Millar-Jones <i>et al.</i> [61]	UK nosocomial outbreak
Govan <i>et al.</i> [62]	Genotypic fingerprinting and extensive epidemiological data provides compelling evidence of person-to-person spread through social contact in and between two UK CF centres
Smith <i>et al.</i> [63]	Further UK outbreak with transmission in clinical and social settings
Bingen <i>et al.</i> [64]	International consensus confirming <i>B. cepacia</i> transmissibility
Corkill <i>et al.</i> [65]	Highlights transmission particularly at UK CF events
Pegues <i>et al.</i> [66]	Demonstration of transmission at USA CF camps
Johnson <i>et al.</i> [25]	Intercontinental spread of Edinburgh/Toronto strain ET12
LiPuma <i>et al.</i> [67]	Inapparent transmission from culture-negative patient (?)
Ryley <i>et al.</i> [68]	Further UK outbreak
Sun <i>et al.</i> [69]	Cable pili demonstrated on intercontinental strain (ET12)
Reverts <i>et al.</i> [70]	Prevalent strain in Belgian clinic
Whitford <i>et al.</i> [71]	Outbreak in UK paediatric clinic
Pitt <i>et al.</i> [46]	Strain ET12 prevalent in UK clinics: accounting for 38% of cases
B. Cases with no evidence of person-to-person transmission	
Glass and Govan [72]	No transmission of pathogenic strain between siblings
Hurdy <i>et al.</i> [73]	No transmission to uncolonised patients during hospitalisation
Taylor <i>et al.</i> [74]	No transmission in UK unit before segregation
Steinbach <i>et al.</i> [75]	No transmission in large CF unit despite no segregation of hospitalised patients

strains as either transmissible and virulent, or non-transmissible and avirulent. There is no scientific justification for this view. In epidemic outbreaks in which patients are colonised by the same strain, some patients may remain asymptomatic whilst other individuals succumb to rapid and unexpected fatal deterioration [37, 62]. In the case of transmission, epidemiological evidence has clearly identified lineages with enhanced transmissibility [25, 46, 62, 69]; however, based on present knowledge, it cannot be stated with confidence that a strain inherently lacks the ability for epidemic spread. Furthermore, apparently 'non-transmissible' strains that have not spread even to a patient's CF sibling have been responsible for fatal infection [72]. Finally, it could be argued that transmission is not strain-dependent, but is associated with nosocomial or social opportunities for transmission. This hypothesis is certainly not supported by the behaviour of the particular *B. cepacia* lineage with a notorious ability to spread in CF centres in the UK [46, 62] and North America [25, 69], referred to as the Edinburgh/Toronto lineage [69] or ET12 intercontinental clone (multilocus enzyme electrophoresis type 12) [25]. For convenience, this particular *B. cepacia* lineage will be referred to as the ET12 lineage in the remainder of this review.

Some CF carers who have experienced transmission of *B. cepacia* amongst small numbers of their patients have argued against segregation on the grounds that no significant clinical deterioration was observed and that implementation of such draconian measures stigmatises patients and prevents valuable social contacts with other CF patients [70]. However, the hypothesis that *B. cepacia* is transmissible but merely a marker of pulmonary deterioration can be chal-

lenged. A recent retrospective study of the clinical status of *B. cepacia*-colonised adults in the 24-month period before colonisation found no difference in their lung function, number of days in hospital or outpatient visits [77]. Furthermore, in numerous case-controlled studies involving large numbers of patients, *B. cepacia* colonisation has been associated in some but not all patients with an accelerated decline in pulmonary function and a poor prognosis [71, 77-81]. Most studies have reported that the risk of clinical deterioration on acquisition of *B. cepacia* is increased in adult patients with severe disease [78-80]. This contrasts with an epidemic outbreak of *B. cepacia* among children, in whom the dominant impact on respiratory function was greater in patients with better levels of respiratory function [71]. Explanations for the range of clinical responses associated with *B. cepacia* colonisation and inability to predict the clinical outcome in individual patients could include: 1, differences in strain virulence; 2, the relatively low 20% 'strike rate' of cepacia syndrome; 3, the influence of co-colonisation by other pathogens; 4, the age at which colonisation occurs; 5, individual host immune responses; and 6, the severity of underlying CF disease.

The hypothesis that *B. cepacia* colonisation is merely a marker of severe lung disease is also undermined by the fact that fatalities have occurred in CF adults with mild CF disease, including individuals not already harbouring *P. aeruginosa* [62]. Finally, one of the most striking results from the first microbiological studies in transgenic CF mice showed that 70% of CF mice exposed to *B. cepacia* succumbed to more severe broncho-pulmonary infection than control animals [82].

The Edinburgh/Toronto/ET12 epidemic lineage

In reviewing the emergence of *B. cepacia* in CF populations in Europe and North America, it is necessary to emphasise the influence of epidemic lineages on the incidence and prevalence of *B. cepacia* within CF centres. Evidence shows that the incidence in a centre can be influenced greatly by the epidemic spread of a single lineage, and that if such spread is discounted then the prevalence of *B. cepacia* in most CF centres remains relatively low at 5–10%. Transient colonisation by *B. cepacia* also influences prevalence and occurs in c. 5% of CF patients; however, transient colonisation is observed very rarely with the ET12 lineage (authors' unpublished observations), perhaps reflecting the high colonisation potential of this clone. From a clinical, epidemiological and evolutionary viewpoint, the influence of this single clone on the CF community is considerable. In the UK alone, it has been isolated in eight (50%) of 16 CF centres and from 68 (38%) of 178 *B. cepacia*-colonised patients [46]. Attempts to identify its origins have been frustrated by a lack of stored isolates; however, investigation of available isolates allows several conclusions to be reached. Based on evidence from MLEE and ribotyping [25] and PFGE [46, 62], the first known isolates of this epidemic lineage were cultured from Ontario paediatric patients in the latter half of the 1980s [25]. In the UK, the first recorded isolate of the same lineage was in August 1989 [62] from a patient who had never been out of the UK nor shown any evidence of *B. cepacia* colonisation during previous bacteriological investigations. The patient had previous contacts with other UK patients colonised by *B. cepacia*, but the isolates from these patients were not available.

From the available evidence, it appears that the Edinburgh/Toronto/ET12 lineage was established in Canada before its appearance in the UK, and that at some stage in the late 1980s, intercontinental spread occurred between UK and Canadian patients whilst attending summer camps in Ontario, followed by inter-regional spread in the UK during social contacts at meetings [25, 62]. It is tempting to conclude that this highly transmissible strain is clonally related to the isolates cultured during the first documented outbreak of *B. cepacia* in CF patients in Ontario, reported in 1984 [37].

Potential pathogenic mechanisms of *B. cepacia*

Although *B. cepacia* produces several putative virulence determinants—including haemolysins, proteases, lipases, siderophores and catalase—a major clinical role for these factors has not been demonstrated convincingly in CF [83, 84]. However, catalase is associated with the organism's ability to resist killing by professional phagocytes and to produce serious infection in patients with CGD [85].

Intracellular survival

Several puzzling clinical and scientific observations have led to speculation that *B. cepacia* can survive and grow within pulmonary phagocytes or respiratory epithelial cells. First, clinical resistance to antimicrobial therapy despite demonstration of an isolate's susceptibility *in vitro*; second, isolation of serum-sensitive isolates in bacteraemic infection [86]; third, chronic pulmonary colonisation despite a pronounced antibody response [87]; and fourth, the close taxonomic relationship between *B. cepacia* and the intracellular pathogen, *B. pseudomallei*. However, to date, the scientific evidence for intracellular survival or growth of *B. cepacia* is not convincing. Studies of intracellularly in bacterial pathogens can be difficult and, in the case of *B. cepacia*, are complicated further by the organism's innate resistance to antibiotics, including aminoglycosides, which are used commonly in intracellular assays to kill extracellular organisms. As it is known that *B. pseudomallei* survives and multiplies within professional phagocytes [88], studies within our group have focused on monocytes, with *Listeria monocytogenes* and *P. aeruginosa* as positive and negative controls, respectively. However, it was not possible to demonstrate either enhanced uptake or survival of *B. cepacia* in monocytes. Previously, Burns [89] reported the observation of *B. cepacia* within CF post-mortem respiratory epithelial cells by electron microscopy, but no further data have been published to validate this important finding. Low-level invasion *in vitro* of a respiratory epithelial cell line by the epidemic ET12 lineage has been demonstrated [90], but the significance of limited epithelial invasion by bacteria remains unclear [91]. A recent and potentially seminal publication has even suggested that enhanced uptake of CF pathogens by epithelial cells expressing surface cystic fibrosis transmembrane conductance regulator (CFTR), followed by epithelial desquamation, may be an important host defence mechanism rather than a bacterial virulence determinant [92].

Overall, the role of intracellularly in the pathogenesis of *B. cepacia* infection in CF patients is still in doubt. As a caveat, the demonstration of its intracellular survival and growth within amoebae raises the possibility that these free-living protozoa may act as an environmental reservoir from which CF patients could acquire the organism [93].

B. cepacia and host immune responses

Colonisation with *B. cepacia* is associated with a pronounced and specific humoral response, including raised serum IgG and IgA and sputum IgA titres against *B. cepacia* lipopolysaccharide (LPS) and outer-membrane protein (OMP) components [87, 94]. Anti-*B. cepacia* antibodies have also been detected in non-colonised CF patients, and particularly in patients colonised with *P. aeruginosa* [87, 95]. Studies with pre-absorbed sera have failed to demonstrate an appreciable

degree of cross-reactivity between the two species, either for OMP or LPS components [87, 96], suggesting that the response to *P. aeruginosa* is not the source of pre-colonisation anti-*B. cepacia* antibody. Generally, levels of anti-*B. cepacia* immunoglobulin in non-colonised patients are low, but the demonstration of substantially raised titres in a subset of patients may reflect previous exposure to *B. cepacia* where an appropriate antibody response has prevented the occurrence of colonisation. On the other hand, the demonstration of antibody in stored pre-colonisation sera from patients who subsequently became colonised, indicates that antibody does not always play a preventative role. Similarly, the role of antibody in patients once they are colonised is unclear; for example, clinical outcome is independent of the magnitude of anti-*B. cepacia* responses [87]. A recent study [97] with immunoblotting techniques has suggested that IgG antibodies against a 30-kDa OMP, identified presumptively as the major immunodominant porin, OMP D [95, 98], are associated with a better prognosis in colonised patients. If these results are confirmed, it raises the possibility of using this OMP as a target for immunotherapy.

The association of *B. cepacia* with CGD, an inherited defect in neutrophil oxidative killing pathways, and the role of neutrophils as the predominant immune effector cell in the CF lung [99], have led to speculation that the interaction between *B. cepacia* and neutrophils may be important in the evasion of host defences by this organism. Speert *et al.* [85] demonstrated that, unlike *P. aeruginosa*, *B. cepacia* is resistant to non-oxidative neutrophil killing mechanisms; hence the role of *B. cepacia* in CGD. Evasion of the normal neutrophil oxidative burst would aid the survival of *B. cepacia* in the presence of a pronounced immune response. Within the CF lung, normal opsonisation processes are compromised severely through the disruption of immune effector molecules by bacterial and host proteases [100, 101]. In particular, cleavage of complement receptors and immunoglobulin molecules within the respiratory tract may neutralise the humoral immune response to *B. cepacia* and enable the organism to persist in the lungs of colonised patients. However, this observation does not explain the ability of rough, LPS-deficient, serum-sensitive *B. cepacia* to cause invasive pneumonitis and septicaemia in patients with elevated anti-*B. cepacia* immunoglobulin titres [86].

Inflammatory damage

Increasing evidence has emerged to suggest that host immune responses are important in the pathogenesis of *B. cepacia* infection. A UK multicentre study has shown that levels of the inflammatory markers, C-reactive protein and neutrophil elastase $\alpha 1$ -antiproteinase complex, are significantly higher during *B. cepacia*-associated exacerbations than in exacerbations

caused by *P. aeruginosa* alone. Aggressive antibiotic treatment with the most active agents available did not eliminate *B. cepacia*, but in most cases was associated with a decline in inflammatory markers to pre-exacerbation levels [102]. In addition, anecdotal evidence indicates that patients who exhibit rapid pulmonary decline and pronounced inflammatory symptoms, but who do not respond to antibiotic therapy, nevertheless respond to treatment with commercial preparations of immunoglobulin. The relative absence of *B. cepacia* antibodies in healthy human donors [87], from whom these immunoglobulins are obtained, suggests that such preparations contain potentially useful anti-inflammatory activity.

An unexpected but informative result from our own studies has demonstrated that LPS from clinical and environmental isolates of *B. cepacia* induces pro-inflammatory cytokines, including the major cytokine tumour necrosis factor α (TNF α), to a level 10-fold that induced by *P. aeruginosa* LPS and matching the inflammatory power of *Escherichia coli* endotoxin [103, 104]. The mechanism involved in *B. cepacia* cytokine stimulation is unclear, but is independent of CD14 receptors. Of interest, induction of TNF α by *B. cepacia* LPS is reduced in the presence of *P. aeruginosa* LPS, suggesting that the diversity of clinical outcomes in patients colonised with *B. cepacia* may be influenced in part by the presence or absence of *P. aeruginosa* and other CF pathogens [105].

What is a true *B. cepacia*?

Further research to establish a gold standard for laboratory identification of *B. cepacia* has assumed high priority. Reliable identification is important not only in attempts to clarify the organism's pathogenic potential, but also because of the clinical, social, psychological and potentially litigious consequences for patients, carers and diagnostic laboratories associated with the organism's acquisition and transmission. Selective media and laboratory protocols for culture and presumptive identification of *B. cepacia* from clinical or environmental sources have been described and their value in microbiological surveillance established [14, 48, 56, 106]. However, existing selective media also support the growth of other gram-negative non-fermenting bacilli [46, 48, 56] and unequivocal identification of *B. cepacia* by multitest commercial systems can present difficulties [44, 56, 106, 107].

There is increasing evidence that organisms presently identified as *B. cepacia* by standard laboratory procedures exhibit such diverse genotypic and phenotypic properties that attempts to generalise on virulence, transmission and antibiotic susceptibility are ill-founded. Simpson *et al.* [44] speculated that epidemic strains may represent a *B. cepacia* sub-

population, arising as bacterial hybrids or through horizontal transfer of virulence genes from the closely related pseudomonads *B. gladioli* and the highly dangerous intracellular pathogen *B. pseudomallei*. Recently, isolates identified as *B. cepacia* were characterised further by analysis of cellular proteins and fatty acid components and clustered by means of computer-assisted numerical comparison of the profiles. Representative isolates from individual clusters were selected to determine genotypic relatedness within and between clusters by means of DNA-DNA and DNA-rRNA hybridisation assays. These molecular phylogenetic studies revealed that organisms identified by conventional tests as *B. cepacia* comprised several new *Burkholderia* spp. [108].

According to taxonomic conventions, new species names should not be given to bacteria that cannot be identified reliably by phenotypic characteristics; instead, such groups can be described by the terms genomovar I, II, etc. [109]. Following this convention, isolates identified as *B. cepacia* by conventional multitest systems such as the API 20NE system (API-bioMérieux, Marcy l'Etoile, France) constitute at least four different genomovars of *B. cepacia*; other presumed *B. cepacia* strains are identified as the nitrogen-fixing organism *B. vietnamiensis*. Preliminary studies on a small number of isolates have indicated that the majority of CF isolates from Belgium and the UK tend to cluster in genomovar III [70, 108]. Subsequent ongoing analyses of a larger collection of environmental, phytopathogenic and clinical isolates in our laboratories have confirmed the potential importance of genomovar identification. For example, the isolate responsible for the first UK report of cepacia syndrome [72], and three individual epidemic clones including the highly transmissible ET12 lineage [25, 44, 62, 69] each belong to genomovar III. It should be stressed that *B. vietnamiensis* and the remaining *B. cepacia* genomovars were also identified amongst isolates from CF patients, and that genomovar III status is not synonymous with high transmissibility [72]. Of the 150 '*B. cepacia*' isolates studied to date, most environmental isolates (including the phytopathogenic type strain ATCC 25416) belong to genomovar I; in contrast, isolates associated with acute clinical decline in CF patients are restricted to genomovar III. These results confirm the complex taxonomic heterogeneity within the genus *Burkholderia* and have important diagnostic implications for infection control in the CF community.

Unique bacterial clones and *B. cepacia* transmission factors

Epidemiological data and genomic fingerprinting suggest that the variable incidence of *B. cepacia*—in particular, the lack of cross-infection in some centres

[75, 81], and the contrasting epidemic spread in others—reflects the behaviour of a relatively small number of highly transmissible clones [46, 69, 110–112].

It seems reasonable to speculate that *B. cepacia* strains responsible for epidemic spread may harbour a common colonising factor whose identification could be exploited for diagnostic and therapeutic purposes. At present, the most significant of these factors is adhesion to respiratory mucin [53, 113–115], associated with giant intertwined fibres referred to as cable pili [53, 114]. The gene responsible for cable pili, *chl*, has been detected in the highly transmissible ET12 lineage, represented by the Edinburgh isolate CF5610 (J2315) [16, 25, 62, 69, 115], and responsible for *B. cepacia* colonisation in 38% of UK patients [46]. In a slightly different form, *chl* has also been associated with epidemic transfer of *B. cepacia* from CF to non-CF patients in a Mississippi outbreak [16, 69, 115]. However, studies with a *chl* DNA probe indicated that *chl* is not present in all epidemic clones, suggesting that other bacterial and host factors need to be identified [69]. Interestingly, a recent study [116] has described enhanced binding of the ET12 lineage to lipid receptors, particularly the galactolipid globotriacylceramide (GB₃), and led to speculation that upregulation of GB₃, mediated through the infection process and TNF stimulation within the lung, may provide an alternative receptor for isolates in which cable pili are poorly expressed and a second receptor system for the epithelial attachment of bacteria that have migrated through the mucosal blanket.

Experimental proof of direct or indirect transmission of epidemic *B. cepacia* is not feasible and can be judged only by circumstantial evidence. However, epidemiological data has strikingly demonstrated such potential. Colonisation with more than one strain of *B. cepacia* is unusual and has been reported in <10% of patients [46]. During the Edinburgh outbreak, PFGE fingerprinting showed that one patient harboured two *B. cepacia* strains in his respiratory secretions, including the ET12 clone; however, only the epidemic strain was transmitted subsequently to his girlfriend [62].

Modes of transmission and the risks of acquisition

The potential risks of *B. cepacia* transmission, either directly by person-to-person spread or indirectly from contaminated fomites, continue to be a major concern to the CF community. Table 2 summarises the extensive documented evidence for direct transmission of *B. cepacia* between CF patients during close contacts within hospitals [61, 63, 65], at educational or summer camps [59, 66] and through other social contacts [62, 63]; in contrast, several reliable studies have found no evidence of cross-infection [72–75]. In

their initial report, LiPuma *et al.* [59] cited previous failures to culture *B. cepacia* from respiratory equipment and environmental surfaces as circumstantial evidence that direct person-to-person spread might be the primary means of transmission. However, a subsequent prospective study [117] with selective culture and DNA-based typing of isolates showed that colonised patients can contaminate their environment; thus indirect transmission might occur *via* contaminated surfaces. The intrinsic resistance of *B. cepacia* to many antibiotics also raised justifiable concern that the use of contaminated home-use nebulisers might present a special hazard for *B. cepacia* acquisition. Currently, evidence for nebuliser-associated transmission is scanty and equivocal. A case-controlled retrospective study of five CF patients undergoing treatment in a CF centre [118] showed a significant association between outpatient nebuliser use and *B. cepacia* colonisation. *B. cepacia* was also cultured from nebulisers used by colonised patients. Unfortunately, no bacterial typing was performed to confirm the clonal relationships of the human and nebuliser isolates. Recently, in a prospective study [119], *B. cepacia* was cultured from three of 35 home-use nebulisers. DNA macrorestriction analysis by PFGE revealed that one of two strains of *B. cepacia* recovered from the nebuliser of one patient was also present in the patient's sputum. However, sputum cultures from the two other patients whose nebulisers harboured *B. cepacia* did not yield the organism, suggesting an environmental origin for the *B. cepacia* strain isolated from the nebuliser. Other studies of nosocomial acquisition of *B. cepacia* in non-CF patients have suggested that respiratory infection probably occurred by indirect transmission following use of contaminated nebuliser devices [31, 120]. Airborne dissemination may also present a small risk of *B. cepacia* acquisition. In a prospective study, *B. cepacia* was recovered from the room air during occupation by five of six patients, but to only a limited extent, with the number of bacteria ranging from 1 to 158 cfu/m³ [121]. Maximum yields were associated with episodes of coughing and, after a patient left the room, the organism persisted in room air for up to 45 min.

To conclude, ethical considerations prevent experi-

ments that could provide scientific data to assess the risks of *B. cepacia* acquisition, including clarification of the frequency of contact and the infectious dose required. Based on accumulated evidence (Tables 2 and 3), skin contact, respiratory aerosols, sharing food, contaminated equipment, co-habitation or undergoing physiotherapy in the same room as a *B. cepacia*-positive individual present reasonable risks of acquisition. However, epidemiological evidence [38, 62], including the high numbers (typically >10⁸ cfu/ml) of *B. cepacia* present in the saliva of colonised patients, suggests that the close and frequent social contact that occurs between siblings, the direct exchange of respiratory secretions associated with kissing, and the involvement of a highly transmissible *B. cepacia* lineage arguably present the greatest risks of acquisition.

Environmental release of *B. cepacia* as a biological control agent

Whilst the CF community debates the clinical issues of *B. cepacia* colonisation and transmission, agricultural microbiologists continue to develop the organism as a biological control agent to exploit its antifungal activity (Fig. 1) for the enhancement of crop yields [122, 123] and its nutritional adaptability in the bioremediation of landfill sites, contaminated soils and ground water aquifers [124–126]. Deliberate environmental distribution of *B. cepacia* as field inoculants raises the issue of the phylogenetic relationship between *B. cepacia* of environmental and clinical origin and the potential hazard for human infection. The debate on this relationship has revealed the gulf that exists between different areas of interest and microbiological expertise and, as stated recently in an editorial comment on another contentious issue, bovine spongiform encephalopathy, 'underscores the weakness of separating agricultural and medical science' [127].

We have stated previously that the scientific evidence that environmental strains of *B. cepacia* present little hazard to man is weak [14] and is based on examination of only a few bacterial isolates and

Table 3. Factors that may influence acquisition of *B. cepacia*

- In colonised individuals, *B. cepacia* saliva counts can exceed 10⁸ cfu/ml, suggesting that the highest risk of patient-to-patient spread is transmission of respiratory secretions during kissing or through sharing of eating or drinking utensils.
- Spirometer mouthpieces become heavily contaminated during lung function tests. Risk avoided by use of disposable mouthpieces. Recovery from the surface of lung function equipment is low.
- Recovery from antibiotic reservoirs of nebulisers has been reported, but incidence is low and the degree of risk appears secondary to the preceding factors.
- Aerosol recovery is low, suggesting low risk of aerosol transmission.
- Hands become contaminated after coughing and the organism can be transmitted by handshake. Survival on hands reduced to 10% after 30 min; this varies in different individuals and may account for variable recovery in surveillance studies.
- Gastrointestinal carriage has not been demonstrated, even in colonised individuals, suggesting that the risk of faecal-oral spread is minimal.
- After surface contamination with *B. cepacia*-containing sputum, viable bacteria can be recovered for several weeks.
- Surface contamination by *B. cepacia* sputum is eliminated by treatment with UV irradiation and with common hospital disinfectants, including Milton, Dettol, alcohol 70%, phenols, iodine and cetrimide. Careful drying is important after washing or disinfection.
- Recovery of *B. cepacia* from soil, plants, drains, lakes and surface waters is low, suggesting that natural environments present a possible but low risk for acquisition.

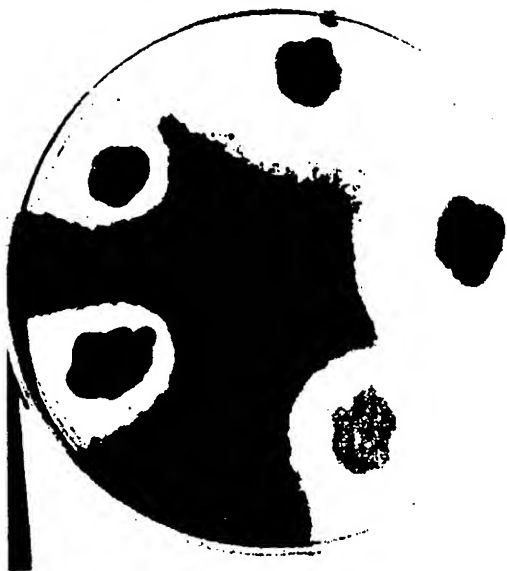


Fig. 1. Inhibition of the phytopathogenic fungus *Rhizoctonia solani* by five isolates of *B. cepacia*. The fungus was inoculated in the centre of the plate and bacteria around the perimeter. Cultures were photographed after incubation for 14 days at room temperature.

inappropriate bacterial properties [24]. Although some studies have indicated that environmental and clinical isolates are distinct, no reliable phenotypic markers have been identified [25, 45, 108]. The suggestion that clinical isolates can be distinguished from soil isolates based on the former's lack of plant pathogenicity [45] is discounted by the fact that CF isolates of *B. cepacia* will readily macerate onion tissue (Fig. 2) [14]. In addition, the invasive *B. cepacia* foot lesions

known as swamp foot [128], acquired by military personnel during jungle training, confirm the pathogenic potential of environmental strains of *B. cepacia* for man.

The potential hazard that some or all environmental *B. cepacia* strains present to the CF community is unclear and requires investigation. The fact that new cases of *B. cepacia* colonisation continue to occur with strains that show no genotypic relationship to other isolates within the same CF centre, points to the environment as a potential source. However, the extent of this risk is difficult to assess. Extensive microbiological safaris into supermarkets and domestic homes [15], and a range of botanical soils and cultivars [14], indicate that *B. cepacia* can be cultured from up to 20% of warm moist environmental sites, particularly soils, but that it is not as ubiquitous as other pseudomonads. Interestingly, in our studies to date, none of the environmental isolates of *B. cepacia* have been identified as belonging to genomovar III.

Conclusions and future prospects

B. cepacia is a striking example of a multiresistant soil saprophyte and phytopathogen that has emerged as an important threat to susceptible human hosts. In the CF community, the degree to which infection control measures should be implemented continues to arouse strong scientific and social debate. The validity of strict control is supported by circumstantial, but nevertheless compelling, evidence for direct person-to-person transmission of epidemic strains through nosocomial and social contact. In contrast, although the risk of indirect iatrogenic spread from contaminated fomites remains

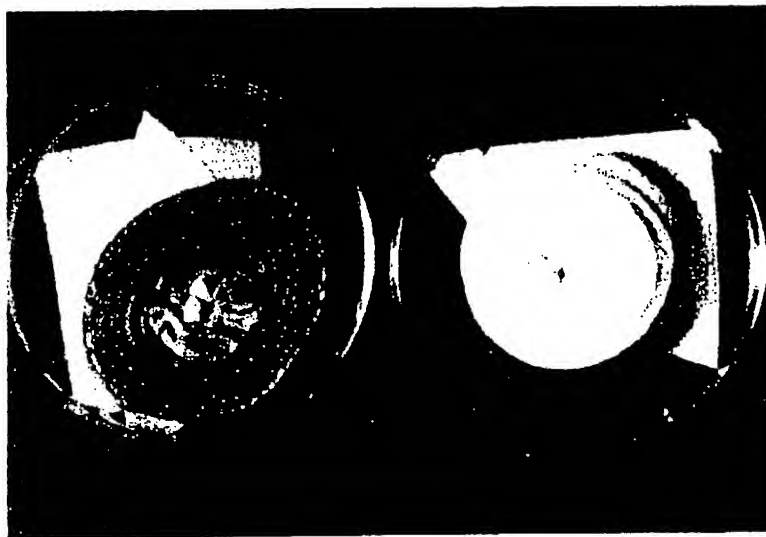


Fig. 2. Soft rot of a segmented 'compromised' onion inoculated with a clinical *B. cepacia* isolate of the epidemic ET12 lineage (left) and an uninoculated control (right), both incubated at 30°C for 72 h. Reproduced with permission from Butler *et al.* [14].

unclear, available evidence suggests that this route is less important than direct transfer. An important caveat in attempts to generalise on *B. cepacia* transmission is evidence that the major epidemics of *B. cepacia* involve a subpopulation of highly epidemic lineages which might be re-allocated ultimately to new species; '*Burkholderia cfe1*' might be an appropriate but probably controversial choice! Ongoing microbiological surveillance in CF centres indicates that sporadic acquisition of epidemic lineages continues to occur when there is a failure to comply with infection control measures. For example, a striking demonstration of the continued potential for transmission of the ET12 lineage was its recent acquisition by an Edinburgh CF adult; extensive inquiries suggested that this patient had social contact for only 10 min whilst visiting another CF male who was hospitalised during an episode of *B. cepacia* septicaemia. Even when infection control appears effective in preventing spread of epidemic lineages, new cases of *B. cepacia* colonisation continue to occur with isolates that exhibit unique PCR ribotyping or PFGE profiles. Such sporadic acquisitions raise a fundamental question concerning the source and colonising potential of individual *B. cepacia* strains. For example, does the environment contain a subpopulation of *B. cepacia* clones that are innately primed for human colonisation, or does colonisation and virulence in man require in-vivo adaptation? Future improvements in laboratory identification of *B. cepacia* subpopulations associated with CF disease and identification of transmission factors, in addition to cable pili, may provide scientific justification for relaxation of segregation in the absence of known epidemic and potentially virulent lineages. Turning our attention to CF patients, we need to clarify why colonisation by the same strain of *B. cepacia* leads to variable clinical responses, ranging from asymptomatic colonisation to rapid fatal pulmonary deterioration. It could be argued that this particular problem is not unique to *B. cepacia*, and that applying Koch's postulates in an attempt to distinguish between sycophancy and pathogenic potential is difficult when dealing with any opportunist pathogen. Certainly, host factors cannot be ignored in attempts to understand the pathogenic processes involved in CF lung infections.

During the final preparation of this review, a deceptively simple and elegant study has illustrated how CFTR-associated defective Cl^- transport across airway epithelia might lead to bacterial colonisation in CF patients. Smith *et al.* [129] showed that the normal human apical epithelial surface is bactericidal for *P. aeruginosa* and *S. aureus*; in contrast, the bactericidal activity was inhibited reversibly in CF epithelia because of a high NaCl concentration. If this phenomenon varies in individual CF patients—or if individual *B. cepacia* strains differ in susceptibility to the defensin-like bactericidal agent—it might explain some of the host- and pathogen-specific anomalies associated with *B. cepacia* pulmonary infection and

suggest novel strategies for infection control and therapy of this unusual and challenging opportunist pathogen.

It is difficult to avoid a final comment on the irony that whilst *B. cepacia* continues to hold the CF community to ransom, agricultural microbiologists seek to develop the commercial and beneficial potential of this microbial Jekyll and Hyde in their search for biological control agents. This situation demonstrates the diversity of microbiology, but should also encourage attempts to reduce the present gulf between agricultural and medical science.

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Note added in proof

Following submission of this review, the results of an epidemiological study of *B. cepacia* in a large series of CF patients attending the CF centre in Verona were published. Cazzola *et al.* concluded that their results are difficult to interpret. Nevertheless, data are essential if progress is to be made in unravelling *B. cepacia* epidemiology, and the results of this study are particularly relevant to the major issues discussed in our review.

Between Nov. 1991 and Dec. 1994, *B. cepacia* was cultured from 85 (11.0%) of 769 CF patients attending the Verona centre. Based on genomic fingerprinting, 32 (53.3%) patients were colonised by individual *B. cepacia* strains; the remaining 28 (46.7%) patients were divided into 10 subgroups, each colonised by a distinct strain. As previously encountered with the ET12 lineage, the outcome of *B. cepacia* colonisation in the Verona study varied from rapidly fatal septicemia to maintenance of reasonably stable respiratory function, even in patients colonised by the same strain. Cazzola *et al.* provide further evidence for hypotheses discussed in our review that some *B. cepacia* strains exhibit a low transmissibility that the environment is a likely source of sporadic new cases: e.g., transmission was observed in only three of eight pairs of CF siblings; in unrelated patients, direct person-to-person transmission was evident in only 10 cases (16.7%); despite a strict segregation policy, whether as in- or out-patients, 15 new colonised patients were identified during 1993. Considering social implications and the paucity of previous data, it was particularly interesting to note that transmission was demonstrated between two unrelated CF schoolmates.

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The identification of *Pseudomonas cepacia* and its occurrence in clinical material

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HOLMES, B. 1986. The identification of *Pseudomonas cepacia* and its occurrence in clinical material. *Journal of Applied Bacteriology* 61, 299-314.

During the 19 year period ending December 1984, 4840 strains of Gram-negative non-fermentative bacteria were submitted to the National Collection of Type Cultures for identification. Of these, 195 strains (4.0% of the total) were identified as *Pseudomonas cepacia* which demonstrates both that the species is regularly encountered in clinical material in the UK and that several laboratories have experienced difficulty in identifying the organism. The sources from which the 195 strains were isolated are reported and also the characteristics by which the species may be recognized. The clinical significance of *Ps. cepacia* is reviewed, and the resistance of this species to disinfectants and antimicrobial agents commonly used to treat *Pseudomonas* infections is discussed to underline the necessity for the precise identification of *Ps. cepacia*.

Pseudomonas cepacia, a phytopathogenic species, was originally described by Burkholder (1950). Ballard *et al.* (1970) considered that *Ps. cepacia* was an earlier synonym of '*Ps. multivorans*' Stanier *et al.* 1966, and as such had nomenclatural priority. There was no phenotypic distinction between *Ps. cepacia* and '*Ps. multivorans*' and although DNA-DNA hybridization experiments supported this view (Ballard *et al.* 1970), a later numerical taxonomic analysis (Sneath *et al.* 1981) of the phenotypic data of Stanier *et al.* (1966) indicated the possibility of some differences between *Ps. cepacia* and '*Ps. multivorans*'. However, all other workers have noted the similarity between *Ps. cepacia* and '*Ps. multivorans*' (e.g. Sands *et al.* 1970) and between these species and strains of the EO-1 group 'eugonic oxidizers, group 1' (King 1964), later called '*Ps. kingii*' by Jonsson (1970; see also Gilardi 1970, 1971; Mackel 1970; Pickett & Pedersen 1970a). Snell *et al.* (1972) established '*Ps. kingii*' (EO-1) as another synonym of *Ps. cepacia* whilst Samuels *et al.* (1973) and Sinsabaugh & Howard (1975) confirmed the synonymy of '*Ps. kingii*' with '*Ps. multivorans*'. Gonzalez & Vidaver (1979) studied

strains of clinical and plant origin and their results also supported the synonymy of *Ps. cepacia*, '*Ps. kingii*' and '*Ps. multivorans*'. *Pseudomonas cepacia* is the correct name nomenclaturally (Palleroni & Holmes 1981).

Pseudomonas cepacia has been reported with increasing frequency in clinical material by workers in the USA and the UK, and with less frequency elsewhere (Table 1). Strains have been isolated from a wide variety of human clinical sources (Table 1) and several reports have appeared detailing the rôle of this organism as the responsible agent in outbreaks of nosocomial infections. In many cases contamination of aqueous topical anaesthetics, disinfectants or irrigating solutions was implicated (Table 1). *Pseudomonas cepacia* is generally regarded as a low grade pathogen (e.g. Hardy *et al.* 1970) but its ability to contaminate and perhaps grow in certain disinfectants in common use in hospitals (e.g. Bassett *et al.* 1970), as well as to grow in deionized or distilled water (Gelbart *et al.* 1975, 1976), makes it a potentially dangerous organism. True infection is seen occasionally (e.g. Bassett *et al.* 1970) but the rôle of the organism has been questioned (Anon. 1970).

Table 1. Summary of previous reports on identification and incidence of *Pseudomonas cepacia*

Country of origin of report	Reference	Common sources of isolates reported (where appropriate)
USA	Sorrell & White (1953)	Blood (endocarditis)
	Schiff <i>et al.</i> (1961)	Blood (endocarditis)
	King (1964, 1967)	Urine, blood, respiratory tract
	Dailey & Benner (1968)	Respiratory tract
	Hardy <i>et al.</i> (1970)	Urine*
	Pickett & Pedersen (1970a, b)	
	Pedersen <i>et al.</i> (1970)	Respiratory tract, blood
	Mackel (1970)	Urinary catheter kits
	Gilardi (1970, 1971, 1972, 1978)	
	Ederer & Matsen (1970)	
	Hugh (1970)	
	Seligman <i>et al.</i> (1971)	Blood (endocarditis)
	Taplin <i>et al.</i> (1971)	Wounds (toe webs)
	Anon. (1972)	Predominantly urine*
	Dixon (1972)	Predominantly urine*
	Ederer & Matsen (1972)	Urine, respiratory tract, wounds, blood
	Gilardi (1972)	Wound, blood, urine
	Schaffner <i>et al.</i> (1973)	Respiratory tract†
	Weinstein <i>et al.</i> (1973)	Respiratory tract
	Dunne <i>et al.</i> (1973)	Blood†
	Anon. (1973)	Blood†
	Meyer (1973)	Blood
	Hamilton <i>et al.</i> (1973)	Blood (endocarditis)
	Rahal <i>et al.</i> (1973)	Blood (endocarditis)
	Neu <i>et al.</i> (1973)	Blood (endocarditis)
	von Gravenitz (1973)	
	Anon. (1974)	Various*
	McKinley & McCroan (1974)	Blood*
	Denney <i>et al.</i> (1975)	Respiratory tract
	Bottone <i>et al.</i> (1975)	
	Noriega <i>et al.</i> (1975)	Blood (endocarditis)
	Feeley <i>et al.</i> (1975)	Respiratory tract
	Matsen (1975)	Predominantly respiratory tract and urine
	Cabrera & Drake (1975)	Blood†
	Gelbart <i>et al.</i> (1975, 1976)	Nebulizer water reservoirs
	Rapkin (1976)	Various†
	Weinstein <i>et al.</i> (1976)	Blood*
	Kaslow <i>et al.</i> (1976)	Blood*
	Frank & Schaffner (1976)	Blood and intravenous fluid*
	Sieber & Fulginiti (1976)	Respiratory tract
	Dixon <i>et al.</i> (1976)	Various*
	Kuehnel & Lundh (1976)	Blood*
	Darby (1976)	Ventricular fluid
	Mandell <i>et al.</i> (1977)	Blood (endocarditis)
	Poe <i>et al.</i> (1977)	Respiratory tract†
	Steere <i>et al.</i> (1977)	Blood†
	Kothari <i>et al.</i> (1977)	Synovial fluid
	Blessing <i>et al.</i> (1979)	Respiratory tract (cystic fibrosis)
	Rhame <i>et al.</i> (1979a, b)	Blood†
	Rosenstein & Hall (1980)	Blood and respiratory tract (cystic fibrosis)
	Berkelman <i>et al.</i> (1981)	Blood*
	Martone <i>et al.</i> (1981)	Various†
	Craven <i>et al.</i> (1981)	Blood*
	Rhame <i>et al.</i> (1981)	Respiratory tract†
	Schmidt <i>et al.</i> (1981)	Respiratory tract
	Rhame & McCullough (1982)	Blood†
	John & Twitty (1982)	Urine*
	Anon. (1982)	
	Berkelman <i>et al.</i> (1982)	Peritoneal fluid

Country of origin of report	
	Decicco <i>et al.</i>
	Lybarger
	Gilardi (1)
	Mackenzi
	Gilligan &
	Rutala <i>et al.</i>
	Styrt & K
	Smith <i>et al.</i>
	Tablan <i>et al.</i>
	Thomasse
	Clegg <i>et al.</i>
UK	Mitchell &
	Burdon &
	Bassett <i>et al.</i>
	Phillips <i>et al.</i>
	Speller <i>et al.</i>
	Snell <i>et al.</i>
	Speller (15)
	Bassett <i>et al.</i>
	Roberts &
	Henderso
	Stirland &
	King & P.
	Pallent <i>et al.</i>
	Nakhla &
Belgium	DeMol (15)
	Zech (1975)
	Yourasov
Australia	Juffs (1973)
	Morris <i>et al.</i>
	Guinness
	Webbing (1)
France	Richard <i>et al.</i>
	Monteil <i>et al.</i>
Canada	Randall (1)
	Gold <i>et al.</i>
	Isles <i>et al.</i>
	McKevitt
	Conly <i>et al.</i>
Switzerland	Pappalard
Chile	Fernandez
Denmark	Bremmelg
Denmark and Holland	Borghans
	Siboni <i>et al.</i>
Israel	Sobel <i>et al.</i>
Japan	Yabuuchi
Sweden	Brauner <i>et al.</i>
Trinidad	Morris & J

* Source traced to contaminated anti coils, pressure transducers and urinary catheters.
† Source traced to contaminated anaesthetic solutions.
The isolate described by Sorrell & White (1953) was established later (Hardy *et al.* 1970).
The isolate described by Schiff *et al.* (1961) was established later (Hardy *et al.* 1970).
The strains of Morris & Roberts (1970) were caused by contamination of 'Ps. multivorans' (Stanier *et al.* 1970).
Several of the cases described by Phil Haigh (1984) were caused by contamination.

(continued)

Identification and occurrence of *Ps. cepacia*

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Table 1 (continued)

Country of origin of report	Reference	Common sources of isolates reported (where appropriate)
sources of isolates reported where appropriate)		
arditis)	Decicco <i>et al.</i> (1982)	Nasal sprays
arditis)	Lybarger (1982)	Urine
respiratory tract	Gilardi (1983)	
tract	Mackenzie & Gilligan (1983)	Respiratory tract (cystic fibrosis)
	Gilligan & Schidlow (1984)	Respiratory tract (cystic fibrosis)
	Rutala <i>et al.</i> (1984)	Blood†
	Styrt & Klempner (1984)	Respiratory tract
tract, blood	Smith <i>et al.</i> (1985)	Bone
ster kits	Tablan <i>et al.</i> (1985)	Respiratory tract (cystic fibrosis)
	Thomassen <i>et al.</i> (1985)	Respiratory tract (cystic fibrosis)
	Clegg <i>et al.</i> (1986)	
arditis)	Mitchell & Hayward (1966)	Urine*
webs)	Burdon & Whitby (1967)	Contaminated disinfectants
ly urine*	Bassett <i>et al.</i> (1970)	Wounds*
ly urine*	Phillips <i>et al.</i> (1971)	Blood†
itory tract, wounds, blood	Speller <i>et al.</i> (1971)	Urine, blood, wounds*
d, urine	Snell <i>et al.</i> (1972)	
tract†	Speller (1973)	Blood (endocarditis)*
tract	Bassett <i>et al.</i> (1973)	Blood*
	Roberts & Speller (1973)	Urine
	Henderson & Byatt (1974)	Vagina, urine, incubator humidifier water*
	Stirland & Tooth (1976)	Wound, central venous pressure line*
arditis)	King & Phillips (1978)	
arditis)	Pallent <i>et al.</i> (1983)	
arditis)	Nakhla & Haigh (1984)	Blood*, †
	DeMol (1979)	Blood*, †
	Zech (1979)	Various*, †
	Yourassowsky <i>et al.</i> (1979)	Blood†
tract	Juffs (1973)	Milk
	Morris <i>et al.</i> (1976)	Contaminated disinfectant
arditis)	Guinness & Levey (1976)	Predominantly urine and blood*, †
tract	Webling (1978)	Ventricular fluid
ly respiratory tract and urine	Richard <i>et al.</i> (1981)	
	Monteil <i>et al.</i> (1981)	Respiratory tract, respirator humidifier water
ter reservoirs	Randall (1980)	Various, predominantly urine*, †
	Gold <i>et al.</i> (1983)	Sputum (cystic fibrosis)
	Isles <i>et al.</i> (1984)	Sputum (cystic fibrosis)
	McKevitt & Woods (1984)	Sputum (cystic fibrosis)
	Conly <i>et al.</i> (1986)	Respiratory tract†
travenous fluid*	Pappalardo <i>et al.</i> (1980)	Blood
tract	Fernandez & Otth (1972)	Blood, cerebrospinal fluid, incubator water
	Bremmelgaard (1975)	
fluid	Borghans <i>et al.</i> (1979)	Blood†
carditis)	Siboni <i>et al.</i> (1979)	Blood†
tract†	Sobel <i>et al.</i> (1982)	Predominantly urinary tract*
	Yabuuchi <i>et al.</i> (1970)	Blood
d	Brauner <i>et al.</i> (1985)	Blood
tract (cystic fibrosis)	Morris & Roberts (1959)	Soil, river water

respiratory tract (cystic fibrosis)

tract†
tract

uid

(continued)

* Source traced to contaminated antiseptic, disinfectant, etc., in some cases *via* such sources as haemodialysis coils, pressure transducers and urinary catheter kits.

† Source traced to contaminated anaesthetics, saline, distilled water, etc.

The isolate described by Sorrell & White (1953) was reported as 'a variant of the genus *Herellea*' but its true identity was established later (Hardy *et al.* 1970).

The isolate described by Schiff *et al.* (1961) was identified as '*Flavobacterium*' but its true identity was established later (Hardy *et al.* 1970).

The strains of Morris & Roberts (1959) were not described as *Ps. cepacia* but were included in the original description of '*Ps. multivorans*' (Stanier *et al.* 1966).

Several of the cases described by Phillips *et al.* (1971) and by Zech (1979), and those described by Nakhla & Haigh (1984) were caused by contaminated blood pressure monitoring equipment.

Over a 19 year period, up to December 1984, 4840 strains of Gram-negative non-fermentative bacteria were submitted to the National Collection of Type Cultures (NCTC) for identification. Of these, 195 strains (4.0% of the total) were identified as *Ps. cepacia* which suggests both that the organism is not uncommon in clinical material in the UK and that many laboratories encounter difficulty in identifying the species. The results of biochemical tests carried out on 195 strains of *Ps. cepacia* are therefore reported here.

Materials and Methods

BACTERIAL STRAINS

One hundred and ninety-five strains of *Ps. cepacia* were examined. They comprised 191 field strains isolated from clinical material, principally in the UK, and sent to the NCTC for computer-assisted identification; the other four strains were reference cultures maintained in the NCTC. The sources of the field strains are given in Table 2. The four reference cultures were NCTC 10743 (type strain) and strains NCTC 10661, 10734 and 10744.

BACTERIOLOGICAL STUDIES

The 191 field strains were identified in some cases solely on the test results provided by the sending laboratory, in other cases on the test results obtained in the sending laboratory combined with the results of further tests done at the NCTC and in the remaining cases solely on the test results obtained at the NCTC. Only the biochemical test results obtained at the NCTC are reported, so that for strains identified solely on the test results obtained in the sending laboratory no results are given. In addition, as differ-

ent numbers of tests were carried out on each strain, the number of strains examined in each test is given in Table 3. The method of computer-assisted identification used in the NCTC has been described elsewhere (Wilkinson *et al.* 1980). The four reference cultures were examined in all the 68 tests listed in Table 3. The methods used for these tests have been described previously (Holmes *et al.* 1975). The test cultures were incubated at 37°C except where otherwise required by the specification for the test.

Results

The strains were Gram-negative rods generally producing non-pigmented colonies on nutrient agar, but 13 (of 173 tested) were yellow pigmented. The biochemical test results are given in Table 3.

Discussion

The biochemical characteristics of the field strains of *Ps. cepacia* (Table 3) agree very closely with those of the reference cultures of the species (which include the type strain) maintained in the NCTC and with the original description of the species given by Burkholder (1950) except that only 30% of the NCTC strains reduced nitrate and 64% produced urease. The biochemical characteristics of all the strains examined also agree well with those presented by other authors. Polymyxin B, colistin and gentamicin are routinely used in the treatment of pseudomonas infections but these agents are not effective against *Ps. cepacia* *in vitro*. Precise identification of the organism thus becomes important, especially as effective therapy may require prolonged or high doses of antimicrobial agents to effect cure. As with

Table 2. Sources of *Pseudomonas cepacia* field strains studied

Source	No. of strains	Source	No. of strains
Blood	42	Catheter tip	14
Transfusion blood	2	Ear swab	2
Respiratory tract	27	Eye swab	2
(sputum, 11)		Anaesthetic	3
(tracheal aspirate or secretion, 15)		Disinfectant	21
(pleural aspirate, 1)		Hospital environment	3
Respirator	21	Miscellaneous	13
Wound swab	12	Unknown	8
Urine	21		

Table 3.

Test
Acid from ASS
glucose
arabinose
cellobiose
fructose
inositol
mannitol
Acid from 10% glucose
Alkali production on
Christensen's citrate
Catalase production
Growth at 37°C
Oxidative in Hugh &
Leifson oxidation-
fermentation test
Tween 20 hydrolysis
Tween 80 hydrolysis
Acid from ASS
adonitol
glycerol
maltose
sorbitol
trehalose
xylose
Growth at room
temperature
Growth on MacConkey ag
Growth on β -
hydroxybutyrate
Growth on Simmons'
citrate
Opalescence on
lecithovitellin agar
Acid from ASS
dulcitol
lactose
Tyrosine hydrolysis
Casein digestion
Acid from 10% lactose
Cytochrome oxidase
production
Growth on cetrimide
Poly β -hydroxybutyrate
inclusion granules
Acid from ASS salicin
Motility at room
temperature
Gelatin hydrolysis
(plate method)
Urease production
Acid from ASS sucrose
Lysine decarboxylase
Gelatin hydrolysis
(stab liquefaction)
Ornithine decarboxylase
Growth at 42°C

Identification and occurrence of *Ps. cepacia*

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Table 3. Biochemical characters of *Pseudomonas cepacia*

Test	Results of reference strains (no. positive/ no. tested)	Results of field strains (no. positive/ no. tested)	Percentage positive of all strains	Sign
Acid from ASS				
glucose	4/4	151/151	100	+
arabinose	4/4	151/151	100	+
cellobiose	4/4	153/153	100	+
fructose	4/4	81/81	100	+
inositol	4/4	128/128	100	+
mannitol	4/4	111/111	100	+
Acid from 10% glucose	4/4	73/73	100	+
Alkali production on				
Christensen's citrate	4/4	128/128	100	+
Catalase production	4/4	167/167	100	+
Growth at 37°C	4/4	162/162	100	+
Oxidative in Hugh &				
Leifson oxidation-				
fermentation test	4/4	162/162	100	+
Tween 20 hydrolysis	4/4	147/147	100	+
Tween 80 hydrolysis	4/4	114/114	100	+
Acid from ASS				
adonitol	4/4	129/131	99	+
glycerol	4/4	113/114	99	+
maltose	4/4	153/155	99	+
sorbitol	4/4	110/111	99	+
trehalose	4/4	146/147	99	+
xylose	4/4	152/153	99	+
Growth at room				
temperature	4/4	162/163	99	+
Growth on MacConkey agar	4/4	129/130	99	+
Growth on β -				
hydroxybutyrate	4/4	150/151	99	+
Growth on Simmons'				
citrate	4/4	156/157	99	+
Opalescence on				
lecithovitellin agar	4/4	79/80	99	+
Acid from ASS				
dulcitol	4/4	136/139	98	+
lactose	4/4	110/113	97	+
Tyrosine hydrolysis	4/4	144/148	97	+
Casein digestion	1/4	80/80	96	+
Acid from 10% lactose	4/4	101/107	95	+
Cytochrome oxidase				
production	4/4	158/166	95	+
Growth on cetrimide	4/4	97/110	89	+
Poly β -hydroxybutyrate				
inclusion granules	1/4	130/145	88	+
Acid from ASS salicin	4/4	96/111	87	+
Motility at room				
temperature	4/4	139/164	85	+
Gelatin hydrolysis				
(plate method)	1/4	122/163	74	d
Urease production	2/4	91/142	64	d
Acid from ASS sucrose	3/4	67/112	60	d
Lysine decarboxylase	3/4	90/168	54	d
Gelatin hydrolysis				
(stab liquefaction)	0/4	57/118	47	d
Ornithine decarboxylase	0/4	64/148	42	d
Growth at 42°C	2/4	49/122	41	d

(continued)

sts were carried out on each
r of strains examined in each
Table 3. The method of
identification used in the
described elsewhere (Willcox et
reference cultures were exam-
8 tests listed in Table 3. The
for these tests have been
isly (Holmes et al. 1975). The
re incubated at 37°C except
required by the specification

Gram-negative rods generally
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(73 tested) were yellow pigment-
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portant, especially as effective
require prolonged or high doses of
agents to effect cure. As with

Strains studied

Source	No. of strains
r tip	14
ib	2
ab	2
retic	3
ctant	21
al environment	3
aneous	13
wn	8

Table 3 (continued)

Test	Results of reference strains (no. positive/ no. tested)	Results of field strains (no. positive/ no. tested)	Percentage positive of all strains	Sign
β -D-Galactosidase production (ONPG test)	3/4	47/122	40	d
Acid from peptone-water- glucose	1/4	52/133	39	d
Aesculin hydrolysis	4/4	39/108	38	d
Motility at 37°C	2/4	59/161	37	d
Nitrate reduction	1/4	42/140	30	d
Acid from ASS raffinose	4/4	21/105	23	d
KCN tolerance	0/4	28/122	22	d
Malonate utilization	0/4	16/122	13	d
Production of yellow pigment	2/4	11/169	8	—
Gluconate oxidation	0/4	6/122	5	—
Growth at 5°C	0/4	6/121	5	—
Acid from ASS ethanol	0/4	1/150	1	—
rhmannose	1/4	0/110	1	—
Arginine dihydrolase	0/4	1/164	1	—
Pigment on tyrosine agar	0/4	2/149	1	—
Arginine desiminase	0/4	0/113	0	—
Deoxyribonuclease production	0/4	0/78	0	—
Fluorescence on King's B medium	0/4	0/109	0	—
Gas from peptone-water- glucose	0/4	0/132	0	—
Hydrogen sulphide production (lead acetate paper)	0/4	0/156	0	—
Hydrogen sulphide production (triple sugar iron agar)	0/4	0/121	0	—
Indole production	0/4	0/120	0	—
Nitrite reduction	0/4	0/126	0	—
Phenylalanine deamination	0/4	0/120	0	—
Reduction of 0.4% selenite	0/4	0/113	0	—
Starch hydrolysis	0/4	0/80	0	—
3-Ketolactose production	0/4	0/78	0	—

The reference strains were NCTC 10661, 10734, 10743 (type strain) and 10744.

+, 85% or more of strains positive; d, 16–84% of strains positive; —, 15% or less of strains positive; ASS, ammonium salt sugar medium; room temperature, 18°–22°C.

other non-fermentative species, insufficient acid is produced in the control tube of the Møller (1955) decarboxylase medium to turn the medium yellow. The unchanged appearance of the control tube can be mistaken for no growth of the strain under test and consequently the decarboxylase tests may be discarded. Decarboxylation of lysine and of ornithine in some strains can thus be overlooked, but positive results are useful diagnostic characters as in clinical material the only other non-fermenter of

clinical interest that decarboxylates lysine is *Ps. maltophilia* and the only other non-fermenter of clinical interest that decarboxylates ornithine is *Alteromonas putrefaciens*.

Esanu & Schubert (1973) recognized three biovars amongst their *Ps. cepacia* strains and Richard *et al.* (1981) recognized eight. Jonsson (1970) found five serogroups and Monteil *et al.* (1981) also found five; O and H serotyping of *Ps. cepacia* has been described by Hejdt *et al.* (1983). Govan & Harris (1985) have described

bacteriocin typing of *Ps. cepacia*. (1981) described mutants blocked of fructose, mannitol and sorbitol. Inhibiting the growth of *Ps. cepacia* in vitro has been described by (1974). Nucleic acid similarities between *Ps. cepacia*, *Ps. mallei* and *Ps. pseudomallei* have been studied by Rogul *et al.* (1977). Petersen (1975) reported the phage typing of *Ps. cepacia* cells following exposure to light; their work suggests a possible contamination in u.v.-treated water.

As well as occurring in soil waters (Morris *et al.* 1959; Taplin *et al.* 1971) where it is able to survive disinfectants. *Pseudomonas cepacia* can also be found in the environment, especially water and surfaces (Bassett *et al.* 1970; Gilardi *et al.* 1971) where it is able to survive disinfectants. *Pseudomonas cepacia* has been found to be inhibited by 0.5% chlorhexidine + 0.5% cetrimide at a 1 in 30 dilution, but large inocula survive even 1 in 30 (Bassett *et al.* 1970). Cells of 10^5 – 10^7 cells/ml have been found in aqueous chlorhexidine alone (Spellman *et al.* 1971) and as many as 10^5 cells/ml have been found in commercial urinary catheter kits containing 0.15% N-alkyl, dimethylbenzyl ammonium chloride in water and phenoxypol (Detergicide; Hardy *et al.* 1970). It also failed to eradicate this organism from blood pressure monitoring apparatus contaminated with heparinized saline (Phillips *et al.* 1971). Nakhla (1984) also noted infections resulting from contaminated blood pressure monitoring apparatus. The source of contamination was deionized water used to prepare E-catheters and disinfecting the transducer cables. *Ps. cepacia* has also been found to be inhibited by benzalkonium chloride (Bassett *et al.* 1970) and to the organomercurial preservative thimerosal (Decicco *et al.* 1971; Nakhla & Haigh 1984). Although isopropyl alcohol and glycerol are effective (Burdon & Whitby 1971; Nakhla & Haigh 1984). It is surprising that infections due to this organism often been initiated by contaminated or antiseptic solutions. This is illustrated in Table 1 where it can be seen that the 191 NCTC field isolates of this organism recovered from disinfectants (Table 1) *Ps. cepacia* can also grow in

bacteriocin typing of *Ps. cepacia*. Allenza *et al.* (1981) described mutants blocked in utilization of fructose, mannitol and sorbitol. Factors influencing the growth of *Ps. cepacia* in sugar solutions have been described by Carson *et al.* (1974). Nucleic acid similarities among *Ps. cepacia*, *Ps. mallei* and *Ps. pseudomallei* have been studied by Rogul *et al.* (1970). Carson & Petersen (1975) reported the photoreactivation of *Ps. cepacia* cells following exposure to u.v. light; their work suggests a possible source of contamination in u.v.-treated waters.

As well as occurring in soil and natural waters (Morris *et al.* 1959; Taplin *et al.* 1971), *Ps. cepacia* can also be found in the hospital environment, especially water and moist surfaces (Bassett *et al.* 1970; Gilardi 1970; Phillips *et al.* 1971) where it is able to survive certain disinfectants. *Pseudomonas cepacia* has been found to be inhibited by Savlon (0.05% chlorhexidine + 0.5% cetrimide) at a 1 in 320 dilution, but large inocula survived dilutions of even 1 in 30 (Bassett *et al.* 1970). Concentrations of 10^5 – 10^7 cells/ml have been found in 0.02% aqueous chlorhexidine alone (Speller *et al.* 1971) and as many as 10^3 cells/ml have been found in commercial urinary catheter kits containing 0.15% N-alkyl, dimethylbenzyl ammonium chloride in water and phenoxyethoxyethanol (Detergicide; Hardy *et al.* 1970). Detergicide also failed to eradicate this organism from blood pressure monitoring apparatus in which contaminated heparinized saline solutions had been used (Phillips *et al.* 1971). Nakhla & Haigh (1984) also noted infections resulting from contaminated blood pressure monitoring apparatus. The source of contamination was deionized water used to prepare Detergicide for disinfecting the transducer cables. *Pseudomonas cepacia* has also been found to be resistant to benzalkonium chloride (Bassett *et al.* 1970; Gilardi 1970) and to the organomercurial preservative thimerosal (Decicco *et al.* 1982) although isopropyl alcohol and glutaraldehyde are effective (Burdon & Whitby 1967; Phillips *et al.* 1971; Nakhla & Haigh 1984). It is not surprising that infections due to this organism have often been initiated by contaminated disinfectant or antiseptic solutions. This is well illustrated in Table 1 where it can be seen that 21 of the 191 NCTC field isolates of this species were recovered from disinfectants (Table 2). *Pseudomonas cepacia* can also grow in either doubly

deionized or doubly distilled water as well as 5% dextrose or 0.9% saline (Gelbart *et al.* 1975, 1976) but it does not grow in intravenous feeding solutions under the usual conditions. Contamination may be easily overlooked as turbidity is not visible (presumably because of the extremely small size of the cells under sub-optimal conditions) even when the organism is grown in distilled water to a concentration of 10^7 /ml (Carson *et al.* 1973). Infections due to this organism can thus also be initiated by contaminated distilled water or intravenous solutions (Table 1).

Pseudomonas cepacia is more resistant than most other Gram-negative bacteria to the commonly used antimicrobial agent benzalkonium chloride. It is also highly resistant to polymyxin B sulphate, another cationic agent that is effective against most Gram-negative organisms. Both agents are thought to act primarily against the cytoplasmic membrane. However, although the work of Adair *et al.* (1976) suggested the cytoplasmic membrane of *Ps. cepacia* to be resistant to polymyxin B (although not to benzalkonium chloride), Manniello *et al.* (1978) believed that resistance to polymyxin B probably involved more than just a barrier effect. The study of Parmelee & Walker (1979), however, suggested that resistance to aminoglycosides (see below) was at the level of the cytoplasmic membrane. Survival for several years in benzalkonium chloride has been reported by Mathews *et al.* (1975), Adair & Geflic (1976) and Geflic *et al.* (1979). The authors of the two latter publications concluded that pharmaceutical solutions containing benzalkonium chloride as an antimicrobial preservative should be formulated without extraneous carbon and nitrogen sources or be preserved with additional antimicrobial agents. Other authors have pointed out (Frank & Schaffner 1976) that in view of the many outbreaks of infection associated with contaminated benzalkonium chloride (Table 1) that such solutions should no longer be used in hospitals. Resistance of *Ps. cepacia* to benzalkonium chloride, as well as to polymyxin B sulphate and chlorhexidine gluconate, has been studied by Richards & Richards (1979). Electron microscopy of the effect of these three agents on the cytoplasmic membrane has been performed by Richards & Cavill (1980). Bassett (1971) reported that while *Ps. cepacia* can survive in Savlon (chlorhexidine gluconate and

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cetrimide) diluted with distilled water it cannot do so when hard tap water is used as the diluent; pH thus has an important influence on the effectiveness of such solutions. Borovian (1983) encountered *Ps. cepacia* in a stored product and found the organism to survive and grow in the formulation even though the pH was less than 3.2. She also found that the strain was able to acquire resistance to two unrelated preservative systems. Adherence of the bacterial cells to glass surfaces appears to afford some protection from chlorhexidine (Pallent *et al.* 1981; Hugo *et al.* 1986).

Pseudomonas cepacia may be recovered commonly from blood (Tables 1 and 2). Septicaemia with endocarditis can occur (Table 1) and in the two earliest reports (Sorrell & White 1953; Schiff *et al.* 1961) the patients died; in the former case, infection may have originated from the urinary tract. There have been several reports of the isolation of *Ps. cepacia* from blood following cardiac surgery (Pedersen *et al.* 1970; Phillips *et al.* 1971; Speller *et al.* 1971; McKinley & McCroan 1974; Weinstein *et al.* 1976). The patients usually recovered spontaneously or with antimicrobial therapy if contamination of the blood pressure monitor or of the intravenous catheter had been the cause of the septicaemia. Two of the NCTC field strains from blood are also known to have been recovered from patients following heart surgery. Endocarditis due to *Ps. cepacia* has been reported in narcotic addicts; one case involved a previously implanted Starr-Edwards mitral prosthetic valve and the other a previously normal aortic valve in a patient with sickle cell anaemia; both cases proved fatal (Seligman *et al.* 1971). The organism has also caused cervical osteomyelitis in an intravenous drug abuser (Smith *et al.* 1985). A case of *Ps. cepacia* endocarditis with ecthyma gangrenosum in a heroin addict was described by Mandell *et al.* (1977). *Pseudomonas cepacia* septicaemia has also been described as the cause of death in two burn patients (Yabuuchi *et al.* 1970; Brauner *et al.* 1985), the several antimicrobial agents administered proved ineffective against the organism in one of the cases (Yabuuchi *et al.* 1970). Two patients who died from bacteraemia both had severe underlying disease (Sobel *et al.* 1982); the use of contaminated chlorhexidine was again the vehicle of infection in these two (and several other) patients.

Pseudomonas cepacia is less frequently associated with wounds (Tables 1 and 2) although reports include nine cases of post-operative wound infection caused by local application of a contaminated disinfectant solution (Savlon Bassett *et al.* 1970). Taplin *et al.* (1971) isolated *Ps. cepacia* from the toeweb of 43/51 (85%) army ranger trainees after swamp training in northern Florida. Within the context of footrot, jungle rot or swamp rot diseases, the authors maintained that there is a clinical entity, associated with *Ps. cepacia*, involving the toeweb and occasionally the plantar surface of the feet, that is characterized by hyperkeratosis, maceration and sometimes induration, inflammation and fissuring. Septic arthritis has arisen following intraarticular injection of contaminated methylprednisolone (Kothari *et al.* 1977).

Pseudomonas cepacia is commonly associated with urinary tract infections (Tables 1 and 2), although in instances where these have been due to contaminated catheter irrigation fluid, infections rarely produced symptoms (Hardy *et al.* 1970; Speller *et al.* 1971) and in many patients with positive urine cultures the urine became sterile spontaneously after catheter removal. In patients with symptoms, however, appropriate antimicrobial therapy did not always eradicate the organism from the urine (Mitchell & Hayward 1966; Roberts & Speller 1973). Contaminated urological instruments have been the vehicle of infection in other hospital outbreaks (Dixon 1972; Anon. 1974). Ederer & Matsen (1972) noted that in 37 of 41 patients from whom *Ps. cepacia* was isolated, prior instrumentation or a manipulative procedure at the site of recovery of the organism strongly suggested that most were nosocomial infections.

Strains of *Ps. cepacia* are also recovered from the respiratory tract or associated equipment (Tables 1 and 2) (Gelbart *et al.* 1975, 1976; Blessing *et al.* 1979) but true infection is rare. One case of true infection was that of a man who developed necrotizing pneumonia after cleaning air conditioners for 3 weeks (Dailey & Benner 1968). Bronchial washings and lung tissue cultures grew *Ps. cepacia*. Therapy was effective with high doses of chloramphenicol (12 g daily). Pneumonitis due to *Ps. cepacia* occurred in this patient and he was found to suffer a phagocyte dysfunction (Denney *et al.* 1975); temperature returned to normal within 36 h of commencing treatment, again with chlor-

ramphenicol 12 g. function was indicated in chronic granuloma and the association syndrome has been described (1975) who described prolonged prophylaxis may well have played a role in colonization and further cases of apparent chronic have been described. Schmidt *et al.* 1976; Clegg *et al.* 1986). Weinstein *et al.* (1976) reported a boy following cardiac surgery did not show a therapeutic response to chloramphenicol (100 mg/kg). A child developed a lung abscess after ultrasonic nebulizer therapy. *Ps. cepacia* (Poe *et al.* 1976) chloramphenicol patient died. Another patient complicated by *Ps. cepacia* septicaemia was described (1980). In fact, recent reports of *Ps. cepacia* septicaemia patients (Table 1) the recovery of urinary tract secretions have been developed (Clegg *et al.* 1986) medium for recovery of the organism. Wu & Thomas (1984) report urinary tract culture of 425 CF patients. *Ps. cepacia* appeared in the culture of the organism. Some of the patients for long time periods with no dramatic improvement, others seemed to have rapid deterioration. Most of the individuals with the disease at the time of colonization it is a chronic infection. The organism is a common pathogen (1984). Tablan *et al.* (1985). *Pseudo-*

frequently associated and 2) although of post-operative application of a solution (Savlon; *al.* (1971) isolated 43/51 (85%) swab training in context of foot rot, cases, the authors, the toe webs and the feet, that otosis, maceration, inflammation and arisen following contaminated methyl-

monly associated (Tables 1 and 2) these have been due to contamination fluid, infections (Hardy *et al.* 1977). In many patients the urine became contaminated after catheter removal. In every, appropriate treatment always eradicate the infection (Mitchell & Peller 1973). Contents have been the hospital outbreaks (Ederer & Matsen 1975). 41 patients from prior instrumentation at the site of strongly suggested infections.

Also recovered from associated equipment (*et al.* 1975, 1976; the infection is rare. was that of a man with pneumonia after 3 weeks (Dailey & Ashings and lung *cepacia*. Therapy was of chloramphenicol due to *Ps. cepacia* he was found to on (Denney *et al.* 1977) to normal within it, again with chlo-

ramphenicol 12 g daily. The phagocyte dysfunction was indistinguishable from that found in chronic granulomatous disease of childhood and the association of *Ps. cepacia* with the latter syndrome has been noted by Bottone *et al.* (1975) who described three such cases. Prolonged prophylactic and antimicrobial therapy may well have played a significant role in the colonization and infection of these patients. Further cases of pneumonia in children with apparent chronic granulomatous disease have been described (Sieber & Fulginiti 1976; Schmidt *et al.* 1981; Styrt & Klempner 1984; Clegg *et al.* 1986). Another case was reported by Weinstein *et al.* (1973) of pneumonia in a young boy following cardiac surgery; the patient did not show a therapeutic response until his daily chloramphenicol dose was increased to 3 g (100 mg/kg). A diabetic patient with pneumonia developed a lung abscess after therapy with ultrasonic nebulization (the reservoir of the nebulizer proved to be contaminated with *Ps. cepacia* (Poe *et al.* 1977)), despite treatment with chloramphenicol for an extended period, the patient died. Another case of cystic fibrosis (CF) complicated by *Ps. cepacia* pneumonia and septicæmia was described by Rosenstein & Hall (1980). In fact, recent years have seen increasing reports of *Ps. cepacia* isolation from cystic fibrosis patients (Table 1). An isolation medium for the recovery of this organism from the respiratory secretions of such patients has indeed been developed (Gilligan *et al.* 1985; a selective medium for recovery of the organism from water solutions and lotions has been described by Wu & Thompson 1984). Gilligan & Schidlow (1984) reported the organism from respiratory tract cultures of approximately 20% of 425 CF patients. They found that recovery of *Ps. cepacia* appeared to be associated with deterioration of the clinical status of some patients. Some of the patients harboured the organism for long time periods (for 6 years in one patient with no dramatic change in his disease) but others seemed to show a progressive and rather rapid deterioration in their clinical condition. Most of the individuals had rather severe lung disease at the time of colonization and once colonized it is almost impossible to eradicate the organism (Gilligan & Schidlow 1984). Similar findings were reported by Isles *et al.* (1984), Tablan *et al.* (1985) and by Thomassen *et al.* (1985). *Pseudomonas cepacia* strains from CF

patients appear to be particularly virulent (Montie *et al.* 1985) and this is possibly associated with the ability of such strains to produce pyochelin (Sokol 1986).

The distribution by source of the strains submitted to the NCTC for identification (Table 2) reflects their incidence in clinical specimens (Table 1). Unfortunately, few clinical details were received with the strains so the literature has to be relied upon for assessing the clinical significance of the organism. However, three of the NCTC strains may have been clinically significant. One strain was isolated on three separate occasions from a thoracic wound following surgery to correct a ventricular septal defect. Another strain was isolated from an intravenous catheter tip and from subsequent blood culture of a 23-year-old man with clinical septicaemia following pulmonary embolus and pneumonia. He had been admitted as a road traffic accident casualty with a wedge fracture of the third lumbar vertebra and lacerations to the legs. *Pseudomonas cepacia* was not isolated from wound swabs, sputum or urine or from aqueous chlorhexidine plus cetrimide used to disinfect wound tissue. The third strain was isolated in pure culture from an infected dog bite wound. In general, *Ps. cepacia* has a low order of pathogenicity. In the outbreak of infection caused by contaminated anaesthetics described by Schaffner *et al.* (1973) clinical disease did not develop in any patient despite the introduction of large inocula, in several cases more than 10 ml of anaesthetic containing 10^5 – 10^{10} bacteria/ml. Similarly, Leyden *et al.* (1980) reported that an inoculum in excess of 10^5 cells is required to produce cutaneous damage even on scarified skin. It can be seen that *Ps. cepacia* poses a serious threat to the patient in relatively few situations despite the fact that few antimicrobial agents are regularly effective.

Strains of *Ps. cepacia* are generally resistant to ampicillin, carbenicillin, cephalothin, colistin, gentamicin, kanamycin, neomycin, polymyxin B, streptomycin and tetracycline (Moody *et al.* 1972; Matsen 1975). In particular, resistance to amikacin, gentamicin and tobramycin has been studied by Moellering *et al.* (1977). Distinct resistance patterns have been observed amongst isolates of the species (Moody *et al.* 1972). Chloramphenicol has been used in systemic treatment (Dailey & Benner 1968; Hardy *et al.* 1970) and the combination of trimethoprim with

sulphamethoxazole has been found synergistic (Seligman *et al.* 1971; Taplin *et al.* 1971; Nord *et al.* 1974) and certainly effective (Moody & Young 1975). The sensitivity of strains to readily maintained levels of trimethoprim and sulphamethoxazole indicates this as the treatment of choice in many cases. The use of this combination for the treatment of *Ps. cepacia* endocarditis has been described by Neu *et al.* (1973) and Seligman *et al.* (1971, 1973). In the endocarditis case described by Hamilton *et al.* (1973) the combination trimethoprim-sulphamethoxazole proved successful after failure with earlier chloramphenicol treatment. Other authors have obtained successful treatment using this combination together with other antimicrobial agents—polymyxin B (Rahal *et al.* 1973; Noriega *et al.* 1975) and kanamycin (Speller 1973). The former authors (Rahal *et al.* 1973) also experienced treatment failure with chloramphenicol. The combination trimethoprim-sulphamethoxazole has also proved successful in the treatment of wound infections (Bassett *et al.* 1970), septicaemia (Phillips *et al.* 1971) and meningitis (Darby 1976). Apparently only one report has been made of susceptibility of some strains to the polymyxins (Moody *et al.* 1972), other authors report resistance in all strains tested, except when used in combination with trimethoprim-sulphamethoxazole (Rahal *et al.* 1973; Noriega *et al.* 1975). In fact, Feeley *et al.* (1975) reported that when an aerosol of polymyxin B was administered to the upper airways of 292 patients in a respiratory-surgical intensive care unit, with the intention of preventing pneumonia due to *Ps. aeruginosa*, eight patients became colonized by *Ps. cepacia* and pneumonia developed in two others. Successful treatment of septic arthritis (resulting from contaminated methylprednisolone) with gentamicin has been reported by Kothari *et al.* (1977). Although cef-tazidime, a new aminothiazole cephalosporin, is very active *in vitro* against *Ps. cepacia* (Gold *et al.* 1983; Santos Ferreira *et al.* 1985; Klinger & Thomassen 1985) it has not proved effective in the treatment of severe respiratory infections, due to this organism, in CF patients (Gold *et al.* 1983). Other newer antimicrobial agents, such as the β -lactams apalcillin, N-formimidoyl-thienamycin, piperacillin, cefotaxime and azlocillin (Santos Ferreira *et al.* 1985) and ciprofloxacin (Klinger & Aronoff 1985) are also active against *Ps. cepacia* *in vitro* as is amikacin

combined with aztreonam, piperacillin or ticarcillin (Aronoff & Klinger 1984). However, the effectiveness of all these in treatment of the CF patient is as yet unknown. *Pseudomonas cepacia* can even use penicillin G as the sole source of carbon and energy (Beckman & Lessie 1979).

Pseudomonas cepacia is not pathogenic for mice and guinea pigs unless very large doses are administered (Sorrell & White 1953; Schiff *et al.* 1961; Jonsson 1970). Stover *et al.* (1983) showed in a burned mouse model, that *Ps. cepacia* can persist in a burn wound for at least 3 weeks; they suggested that this may provide a model for the study of persistent colonization and infection in a compromised host. Anwar *et al.* (1983) found that *Ps. cepacia* strain NGIC 10661, when grown under different specific nutrient depletions in batch culture, showed varying degrees of susceptibility to engulfment and killing by human polymorphonuclear leucocytes and to killing by human serum. There was also an increase in resistance to killing by whole blood with decrease in temperature. In all cases whole blood was 6–10 times more effective than serum alone in killing the bacterial cells at 37°C.

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Linkage Analysis of Geographic and Clinical Clusters in *Pseudomonas cepacia* Infections by Multilocus Enzyme Electrophoresis and Ribotyping

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Multilocus enzyme electrophoresis and ribotyping were used to characterize 83 strains of *Pseudomonas cepacia*, mostly isolated from cystic fibrosis (CF) patients, although a number of isolates from non-CF nosocomial infections and reference environmental strains were represented. Twenty enzyme electrophoretic types (ETs) were determined; of these, one clone (ET12) was associated with six of nine ribotypes (RTs) said to be geographically representative of the United Kingdom and all of the Ontario (Canada) isolates from CF patients. This clone was not associated with nosocomial infections or environmental strains and was never found in CF isolates from British Columbia or Nova Scotia, Canada, or a center in the eastern United States. Individual isolate *EcoRI* RT signatures did not cluster geographically as did the ET signatures by clonal analysis. Frequently RTs occurred in more than a single ET. Known point source focal nosocomial outbreaks were typified by single ETs and stable RTs. Dendrographic analysis of the strains grouped those strains from CF patients, nosocomial outbreaks, and environmental sources into separate ET families, and diversity analysis indicated that, with the exception of ET17, CF isolates clustered in unique and closely related ETs different from those from nosocomial and environmental sources. This study has also shown the potential of multilocus enzyme electrophoresis to monitor the intercontinental spread of *P. cepacia* strains in CF patients, and this may have a significant impact on plans for CF patient summer camps and design of infection control practices. Whether the intercontinental ET12 clone, which predominates in the United Kingdom and the province of Ontario, linked by summer camp acquisition, has increased virulence for CF patients remains to be established.

Recognition by Toronto, Canada, investigators in 1984 (13) that *Pseudomonas cepacia* was an important pathogen in cystic fibrosis (CF) disease has been amply confirmed (7, 9, 11, 16, 28, 30, 31). Early studies (4, 17) of transmission and risk factors incriminated person-to-person transmission and nosocomial acquisition, while later evidence showed that socially and community-acquired infections were of substantial importance (10, 29). At present, there are more studies suggesting person-to-person transmission of *P. cepacia* than of *Pseudomonas aeruginosa* (10, 17, 23). Investigations of transmission and of clonal virulence have been limited because the usually applied techniques of fine identification such as genomic fingerprinting by restriction fragment length polymorphisms, although of considerable value in outbreak investigations, have not in this regard been sufficiently discriminatory (1, 19, 29). Although of value in rapid screening of strains for gross genomic similarities or differences, random amplification fingerprinting (2) is limited in that it lacks reproducibility and is not sufficiently reliable or robust to evaluate clonal diversity. To date, *EcoRI* ribotyping has been generally accepted as the best method available for typing of *P. cepacia* for epidemiological purposes, and length polymorphisms observed after PCR amplification of the 16S-23S intergenic spacer regions have been suggested as an alternative or adjunct to ribotyping (14, 15). We report

here the use of multilocus enzyme electrophoresis (MLEE) to determine enzyme profiles for 83 strains of *P. cepacia* that differ widely in their geographic distribution and clinical and environmental origins. MLEE was found to clearly differentiate isolates according to origin and clinical source to a degree not obtained with *EcoRI* ribotyping or other previous methods.

MATERIALS AND METHODS

Bacterial strains. Sources of *P. cepacia* strains used in this study are summarized in Table 1. We selected strains from CF patients who were geographically widely distributed and also included some strains from both nosocomial outbreaks and the environment. The isolates from CF patients in Vancouver ($n = 14$), Ottawa ($n = 10$), and Halifax ($n = 18$), Canada, were collected over a period of 9, 2, and 7 years and were isolated from 12, 2, and 3 patients, respectively. American Type Culture Collection (ATCC) reference strains were included for reference purposes. The identification of all strains as classical *P. cepacia* was confirmed by fatty acid methyl ester analysis (8, 22) and conventional biochemical phenotypic characterization prior to inclusion in the study. Isolates were frozen in glycerol-peptone at -70°C for prolonged storage and used as required.

Preparation of enzyme extracts. *P. cepacia* isolates from storage were grown overnight at 37°C on heart infusion agar plates (Difco Laboratories). Cultures were harvested by scraping the growth from one petri dish (150 by 15 mm) per strain and suspending the cells in 2 ml of 10 mM Tris-1 mM EDTA-0.5 mM NADP (pH 6.8). Cells were lysed with a Braun-sonic 1510 sonicator (B. Braun Melsungen AG) with three 10-s sonication pulses, each followed by at least 1 min of

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TABLE 1. Clinical associations and geographic sources of *P. cepacia* isolates

Clinical association or source	Geographic location	No. of strains	Abbreviation
CF patients	Canada		
	Vancouver, British Columbia	14 ^a	CF-VAN
	Toronto, Ontario	4	CF-TOR
	Sudbury, Ontario	1	CF-SUD
	Ottawa, Ontario	10 ^a	CF-OTT
	Halifax, Nova Scotia	18 ^a	CF-HFX
	United States (Pittsburgh)	4 ^b	CF-PITT
	United Kingdom and Ireland ^c		
	Birmingham	1	CF-BIRM
	Belfast	2	CF-BELF
	Edinburgh	1	CF-EDIN
	London	2	CF-LOND
	Manchester	1	CF-MANC
	Dublin	2	CF-DUBL
Strains from CF patients used for serotyping	Canada and United States ^d		
	Toronto (serotype A)	1	CF-STA
	Cleveland (serotype B)	1	CF-STB
	Calgary (serotype C)	1	CF-STC
	Cleveland (serotype D)	1	CF-STD
	Toronto (serotype E)	1	CF-STE
Nosocomial outbreak	Ottawa (neonatal nursery)	6	NS-OTT
	Pittsburgh	9 ^e	NS-PITT
ATCC reference strains	Bronchial washings (ATCC 25609)	1	ATCC-HUM
	Onion (ATCC 25416)	1	ENV-ONIO
	Forest soil (ATCC 17759)	1	ENV-SOIL

^a Some isolates in these groups were collected from the same patients over extended time periods and were obtained courtesy of D. Haase (Halifax), D. Speert (Vancouver), and N. MacDonald (Ottawa).

^b Strains received from D. Speert, provided by T. Stull and representative of the RTs as previously described (17).

^c Strains received courtesy of M. Kaufmann, Central Public Health Laboratory, Division of Hospital Infection, Colindale, London, and representative of typical United Kingdom and Ireland RTs.

^d Strains received from D. Woods, representative of serotypes A to E (21).

^e Strains received from J. LiPuma, representative of the seven outbreaks previously described by Rabkin et al. (24).

ice bath cooling. Each sample was centrifuged in a 1.5-ml microcentrifuge tube for 20 min at 12,000 × *g* in an Eppendorf refrigerated centrifuge. The supernatant was filtered with 0.2-μm-pore-size sterile Acrodisc low-protein-binding nonpyrogenic membrane filters (Gelman Sciences). Aliquots (1 ml) were stored in vials at -70°C.

Electrophoretic enzyme typing. Electrophoretic analysis of enzymes and subsequent staining procedures were performed as described by Selander et al. (26) and Carson et al. (5). Starch was purchased from Connaught Laboratories Ltd., Willowdale, Ontario, Canada. The following enzymes were assayed: adenylate kinase (ADK; EC 2.7.4.3), esterases (EST; EC 3.1.1.1), fumarase (FUM; EC 4.2.1.2), glutamic-oxalacetic transaminase (GOT; EC 2.6.1.1), glucose-6-phosphate dehydrogenase (G6P; EC 1.1.1.49), 3-hydroxybutyrate dehydrogenase (HBD; EC 1.1.1.30), isocitrate dehydrogenase (IDH; EC 1.1.1.42), 6-phosphogluconate dehydrogenase (6PG; EC 1.1.1.44), phosphoglucose isomerase (PGI; EC 5.3.1.9), and malate dehydrogenase (MDH; EC 1.1.1.37). Electromorphs (alloenzymes) of each enzyme were numbered by decreasing anodal mobility and were equated with alleles at the corresponding structural gene locus. Distinctive combinations of alleles over the 11 enzyme loci (multilocus genotypes) were designated electrophoretic types (ETs). Simultaneous double-allele banding patterns observed for HBD and IDH were scored independently and designated HBT (top), HBB (bottom), and IDH (top).

Ribotype (RT) analysis. *P. cepacia* was cultured for DNA extraction as described above. Cells were harvested into TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and lysed by the addition of sodium dodecyl sulfate and proteinase K

(Boehringer Mannheim) to 0.5% and 50 μg/ml, respectively, and incubation at 37°C for 30 min. DNA was extracted and quantified by standard techniques (25) and resuspended in TE buffer plus 10 mg of RNase (Boehringer Mannheim) per ml. DNA (5 μg) was digested with 50 U of *Eco*RI in accordance with the manufacturer's specifications (Boehringer Mannheim) and analyzed by electrophoresis using 0.8% agarose gels in TAE buffer (40 mM Tris-20 mM acetic acid-1 mM EDTA) with ethidium bromide (10 μg/ml). In each gel, *Hind*III DNA fragments of λ (GIBCO BRL) were used as migration references. Size-separated restriction fragments were transferred to a Hybond-N+ membrane (Amersham) with a PosiBlot pressure blotter (Stratagene) and then fixed to the membrane by cross-linking using a UV Stratalinker (Stratagene). The 7.5-kb *Bam*HI fragment of plasmid pKK3535 containing the *E. coli* *rrnB* gene was used as a probe. The *E. coli* strain harboring pKK3535 was received through the courtesy of M. Altwegg, Department of Medical Microbiology, University of Zurich (20). The probe was randomly labelled with digoxigenin-11-dUTP using the DIG DNA Labelling and Detection Kit (Boehringer Mannheim). Hybridization was performed at 65°C following methods recommended for Hybond-N+ using approximately 50 ng of labelled probe. Chemiluminescence detection procedures were performed as described in the DIG DNA Labelling and Detection Kit. The rRNA gene fingerprints of isolates were visually scored and given numerical designations for each unique RT.

Statistical analysis. Genetic diversity (*h*) at a given enzyme locus among either ETs or isolates was calculated from the allele frequencies among ETs or isolates as $h = (1 - \sum x_i^2)/(n-1)$, where x_i is the frequency of the *i*th allele and *n* is the

TABLE 2. Clonal allelic profiles and frequency of isolation of strains belonging to 20 ETs of *P. cepacia*

ET	No. of isolates (total = 83)	Strain(s) clustering in ET group (no. of isolates) ^a	Electrophoretic position of allele at enzyme locus:											Strain signature <i>EcoRI</i> RT(s) (no. of isolates)
			HBT	HBB	IDH	MDH	6PG	G6P	GOT	ADK	EST	FUM	PGI	
1	1	CF-STB	0	2	5	1	2	1	3	3	6	1	3	17
2	1	CF-VAN	0	2	7	1	2	3	4	1	0	1	1	24
3	1	NS-PITT	0	2	7	1	3	3	5	1	0	1	1	24
4	2	CF-VAN, CF-BIRM	1	2	1	1	4	1	3	5	0	2	5	18, 20
5	1	CF-VAN	1	2	1	2	4	1	3	5	0	2	5	26
6	3	CF-VAN	2	1	1	1	4	1	3	5	0	2	5	19 (3)
7	1	CF-LOND	2	2	1	1	2	1	3	5	0	2	5	20
8	2	NS-PITT	2	3	3	1	2	2	1	4	6	1	2	30, 31
9	1	ENV-SOIL	3	2	2	1	3	1	1	3	4	2	3	10
10	1	NS-PITT	3	2	5	1	2	1	3	3	6	3	1	34
11	5	NS-OTT (2) NS-PITT (2) CF-STC (1)	3	3	6	1	4	3	3	4	3	1	3	7 (2) 32, 33 7
12	22	CF-ONT (15) CF-UK (6) CF-STA (1)	4	2	2	1	2	1	3	3	2	2	3	1 (2), 2, 4 (10), 15, 23 1, 2, 3, 4 (2), 5 5
13	1	CF-VAN	4	2	2	1	2	1	3	3	6	2	3	22
14	1	CF-DUBL	4	2	5	1	1	1	2	3	1	2	3	25
15	1	NS-PITT	4	2	5	1	2	1	3	1	1	2	1	12
16	18	CF-HFX	4	2	5	1	2	1	3	2	1	2	1	12 (14), 27, 28, 29 (2)
17	12	CF-VAN (7) CF-STD and CF-STE (2) NS-PITT (2) ATCC-HUM	4	2	5	1	2	1	3	3	1	2	1	11, 12 (4), 13, 14 12, 16 12 (2) 8
18	4	NS-OTT	4	2	5	1	2	1	3	3	1	2	3	6 (4)
19	1	ENV-ONIO	4	4	2	1	2	1	2	3	5	2	3	9
20	4	CF-PITT	5	4	4	1	2	1	4	5	0	2	4	21 (4)

^a See Table 1 for abbreviations.

number of ETs or isolates (26). Mean genetic diversity (H) is the arithmetic average of the h values for all of the loci. Genetic distance was expressed as the proportion of mismatched loci between pairs of ETs, and null alleles were excluded from the calculation of pairwise distances. The multilocus diversity analysis and dendrogram based on the average-linkage algorithm were generated by using ETDIV and ETCLUS programs (version 2.3) provided through the courtesy of T. S. Whittam, Institute of Molecular Evolutionary Genetics, Pennsylvania State University. Linkage disequilibrium analysis was performed by using the ETLINK component of this analysis software.

RESULTS

In the collection of 83 strains of *P. cepacia* described in Table 1 and analyzed by MLEE and *EcoRI* ribotyping, 66 were isolates from sputum samples from chronically colonized CF patients and included 5 isolates used to develop a differential serotyping scheme (21). Of the other 18 strains, 15 came from point source nosocomial infections and 3 were ATCC strains, with 2 originating from the environment and 1 from a human source. To our knowledge, only the CF isolates used for serotyping had been previously examined by MLEE (5). Some of the isolates which were kindly provided by other laboratories had been categorized by these sources as having RTs that were representative of the strains isolated in their geographic area.

Of the 14 enzymes originally described for *P. cepacia* MLEE analysis (5), 10 showed differences in electrophoretic mobility that were readily interpretable and polymorphic for a range of two to seven alleles. Although single alleles were observed for most enzymes in this MLEE analysis, two alleles were detected

simultaneously for both HBD and IDH in almost all isolates and double-allele banding patterns were scored for HBD (HBT and HBB). Only one of the IDH alleles was sufficiently polymorphic to warrant statistical analysis. Initially aconitase, phosphoglucosmutase, alkaline phosphatase, and acid phosphatase were included in the enzymes analyzed, but these were eliminated on the basis of banding patterns that were poorly defined and difficult to interpret. Peptidases, malic enzyme, and aspartate dehydrogenase were chosen as possible replacements but failed to produce consistent recordable banding patterns for the majority of isolates. Thus, 11 enzyme loci were scored for 10 polymorphic enzymes as specified earlier.

Enzyme profiles of the 83 *P. cepacia* isolates allowed the identification of 20 ETs, for which the strain source, *EcoRI* RT, and individual allelic profiles are shown in Table 2. The average number of alleles per locus for all isolates and the three categories of strains or populations is presented in Table 3 and varies from 4.64 for the total number of isolates to 1.45 for the environmental strains. The reference strains used for serotyping were analyzed as CF isolates for assignment of population category but have been designated CF-STA to CF-STE to assist in the interpretation of results. ET diversity for the total population and within each major category ranged from 0.780 for CF samples to 0.795 for nosocomial samples and 0.848 for all ETs as indicated in Table 3. The mean genetic diversity within 20 ETs and within 83 isolates (H) also appears in Table 3 for the total population of strains and the three categories. Among the ETs in which CF isolates clustered, six were favored by multiple isolates (2 to 22); consequently, there was less genetic diversity, on average, among isolates ($H = 0.343$) than among ETs ($H = 0.558$). This observation was consistent with the greater genetic diversity among ETs in the total population ($H = 0.581$) compared with that among

TABLE 3. Analysis of the genetic diversity among multilocus ETs and isolates of *P. cepacia* by using allelic differences at 11 enzyme loci

Population	No. of ETs	No. of isolates	Polymorphic loci	Mean no. of alleles	ET diversity ^a	Genetic diversity among:			
						ETs		Isolates	
						<i>H</i> ^b	SE	<i>H</i> ^b	SE
Total	20	83	1.00	4.64	0.848	0.581	0.069	0.408	0.072
CF	13	66	1.00	3.82	0.780	0.558	0.072	0.343	0.079
Nosocomial	7	15	0.91	3.00	0.795	0.606	0.070	0.543	0.062
Environmental	2	2	0.45	1.45	0.000	0.455	0.157	0.455	0.157

^a Estimates probability that two isolates chosen at random from within a population are of different ETs.

^b Mean genetic diversity (*H*) is the arithmetic average of *h* values for all loci calculated by $h = (1 - \sum x_i^2)/(n/n - 1)$. Details of *h* values for individual enzyme loci are given in Table 4.

isolates (*H* = 0.408). Since most of the ETs which represented nosocomial and environmental isolates contained only single isolates, there was less difference in genetic diversity between ETs and isolates for these two population subsets. For the total population, single-locus diversity (*h*) was high for IDH (0.821), EST (0.805), HBT (0.800), PGI (0.742), and ADK (0.737). This pattern was similar for isolates from CF patients, and some differences were observed in frequencies for G6P and FUM for nosocomial isolates (Table 4). Linkage disequilibrium (*D'*) calculated for each pair of alleles at two loci and the frequency tabulated over all pairs of loci resulted in a U-shaped distribution from -1.0 (*D'* = 0.606) to +1.0 (*D'* = 0.218). This distribution of *D'* values is typical for *Escherichia coli* populations and is the consequence if most pairs of alleles are in complete association (3, 12).

ETs 12, 16, and 17 were most represented among our ET groups, containing 22, 18, and 12 isolates, respectively (Table 2). An analysis of a much larger number of isolates would have to be examined to establish whether these ETs predominate in all clinical isolates of *P. cepacia*. Without exception, however, all of the isolates found to cluster by MLEE in ETs 12 and 16 originated from CF patients and included the serotype A reference strain originally isolated from sputum of a CF patient in Toronto. The serotype A strain shared a common RT (RT5) with one of the representative strains from London, United Kingdom. ET17 was more diverse and clustered isolates from CF patients in Vancouver with nosocomial strains from the United States, two of the serotyping strains, and the

ATCC reference strain from bronchial washings. Serotyping strains D and E clustered in ET17; originated from Cleveland, Ohio, and from Toronto; and were RT16 and RT12, respectively, in this study.

Three genetically similar families of ETs were observed for CF isolates with a genetic distance of ≤ 0.200 (Fig. 1) and included ETs 4, 5, 6, and 7 with 7 isolates from Vancouver, Birmingham (United Kingdom), and London; ETs 12 and 13 with 23 isolates from Canada, the United Kingdom, and Ireland; and ETs 15, 16, and 17 with 31 isolates from Halifax, Vancouver, Cleveland (serotype D), and Toronto (serotype E). All strains in this study from Ontario (Canada) clustered in ET12 in addition to the representative strains from Manchester, London, and Edinburgh in the United Kingdom; two strains from Belfast, United Kingdom; and one from Dublin, Ireland. This ET is characterized by a unique EST-2 allele which may be a marker for the predominant clone in the United Kingdom-Ireland and Ontario.

In the total population of 83 strains, 34 *EcoRI* RTs were identified as indicated in the last column of Table 2. An observation that may have significance is that the ETs that contained strains from a variety of CF patients consistently demonstrated a greater diversity of RTs than did ETs containing predominantly nosocomial isolates. In addition, the same RT was frequently observed to occur in more than a single ET (Fig. 1) as follows: RT24 in ETs 2 and 3, RT20 in ETs 4 and 7, and RT12 in ETs 15, 16, and 17. These data suggest that isolates from ETs 3 and 15 have high potential for nosocomial spread to CF patients. Of all the CF isolates characterized by MLEE, only ET20, comprising four strains from the CF center in Pittsburgh, Pa., diverged from the other clusters at a genetic distance of more than 0.60. These results may be indicative of nosocomial or environmental acquisition in this particular patient population. In this study, the majority of ET12 isolates from Ontario CF patients were of RT4 and identical to the RTs for Manchester and Dublin, and the single Edinburgh isolate, although ET12, was identified as RT3.

DISCUSSION

Analysis of the data from MLEE and *EcoRI* ribotyping of our *P. cepacia* isolates demonstrated three main features. First, by MLEE, *P. cepacia* found in the environment is demonstrably quite different genomically from pulmonary and nosocomial isolates and unlikely to be responsible for infections in CF patients. Since the number of environmental strains we examined is underrepresented in this study, additional strains would need to be studied to validate this conclusion. Second, isolates of *P. cepacia* colonizing CF patients attending the same clinic almost always have the same ET and frequently different RTs. This strongly suggests that this pathogen is readily transferred

TABLE 4. Genetic diversity at 11 enzyme loci for all *P. cepacia* strains and three populations

Allele	Single-locus diversity (<i>h</i>) of:			
	Total population	CF samples	Nosocomial samples	Environmental samples
HBT	0.800	0.833	0.810	1.000
HBB	0.437	0.423	0.476	1.000
IDH	0.821	0.833	0.714	0.000
MDH	0.100	0.154	0.000	0.000
6PG	0.553	0.564	0.524	1.000
G6P	0.353	0.282	0.667	0.000
GOT	0.574	0.410	0.524	1.000
ADK	0.737	0.744	0.762	0.000
EST	0.805	0.756	0.810	1.000
FUM	0.468	0.385	0.714	0.000
PGI	0.742	0.756	0.667	0.000

H^a (SE) 0.581 (0.069) 0.558 (0.072) 0.606 (0.070) 0.455 (0.157)

^a Mean genetic diversity (*H*) is the arithmetic average of *h* values for all loci calculated by $h = (1 - \sum x_i^2)/(n/n - 1)$.

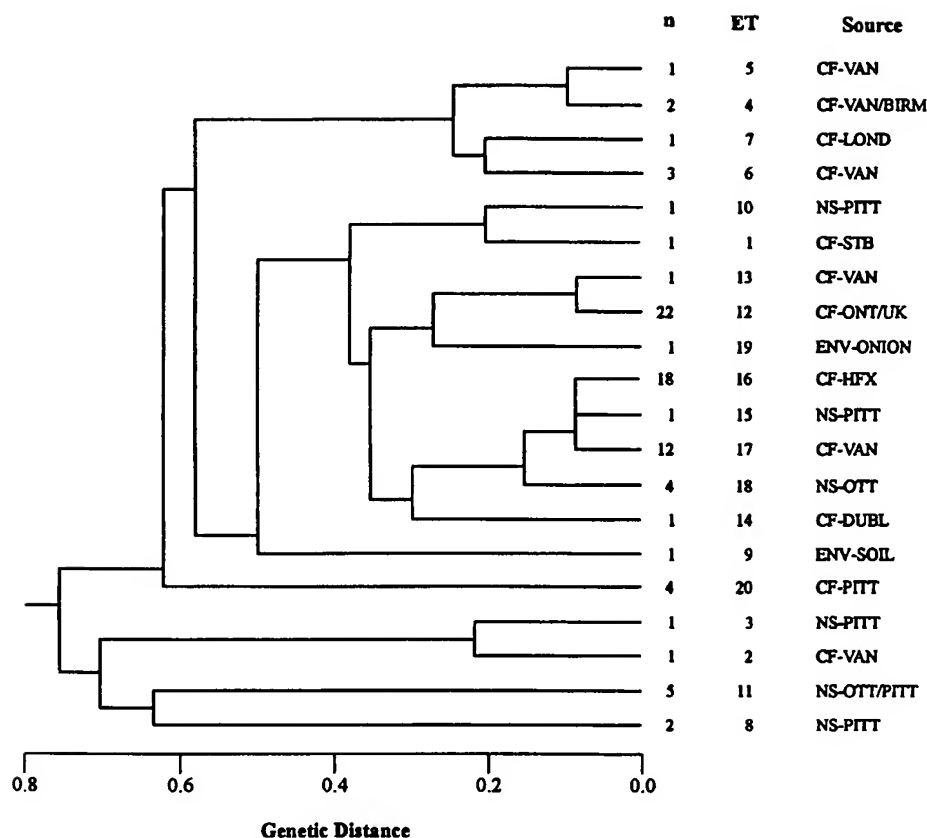


FIG. 1. Genetic relationships among 83 strains and 20 ETs of *P. cepacia* isolated from CF patients, nosocomial infections, and environmental sources. ETs are numbered as assigned after multilocus diversity analysis (ETDIV) based on 11 enzyme loci, and the dendrogram resulted from a cluster analysis (ETCLUS) of genetic distances by the average linkage algorithm using statistical analysis programs (version 2.3) provided by T. S. Whittam. The number (n) of isolates is indicated for each ET. See Table 1 for source abbreviations.

patient to patient, especially in social settings as recently documented (10, 29). Third, cluster analysis of isolates from particular geographic areas groups them into the same ETs, even when their RT profiles are widely different. Geographic areas appear to nurture unique clones of *P. cepacia*, and ET analysis clearly groups these strains.

The stable characteristics of MLEE allowed the clear demonstration of intercontinental spread. The Ontario isolates from CF patients which we grouped as ET12 were identical to many isolates from the United Kingdom but different from the representative strains from the eastern United States (ET20) and both the Atlantic and Pacific coasts of Canada. This was, in part, not unexpected since a recorded epidemic of a highly transmissible strain in the United Kingdom (10) included two colonized patients who were said to have spent time in an Ontario summer camp in September 1990. In our study, both the Manchester isolate and the majority of the Ontario CF isolates were not only of identical ET but also of identical RT (RT4). The Edinburgh isolate, although ET12 and considered part of the same endemic cluster, was RT3. Of some interest is the fact that a few of the ET12 strains from Ontario pediatric CF patients were isolated in 1987 and 1988 and therefore appear to have been established in Ontario prior to 1990. Intercontinental spread of this putative virulent clone may predate the camp of 1990 that is said to have been the source of the United Kingdom outbreak (10). Although we have no additional information on this feature, we surmise that there

may have been summer camps in earlier years at which contact and transmission occurred. Additional investigations will be required to establish the initial date and direction of spread of *P. cepacia* (ET12) between the United Kingdom and Ontario. These circumstances suggest that camps attended by CF patients in regions far removed from their own communities represent a risk of infection with different *P. cepacia* strains, some of which may have enhanced virulence.

It is our general conclusion that MLEE provides data on clonality which are stable and capable of delineating families of strains typical of geographic and regional *P. cepacia* populations, uninfluenced by trivial and ephemeral factors. In this respect RT analysis is unsatisfactory, being sensitive to selective pressures from the patient and the intimate environment. Used together, however, these techniques provide the degree of discrimination required of serious demographic and epidemiological studies. This approach has been previously recommended (6) for the analysis of *P. aeruginosa* isolates from chronic lung infections in CF patients.

Finally, with respect to available techniques, in our experience with *P. cepacia*, the technique which identifies most sensitively the highest level of genetic drift is neither ET nor RT analysis but is restriction fragment analysis using pulsed-field gel electrophoresis after restriction endonuclease digestion with specific enzymes which cleave infrequently. Choice of these enzymes is critical to ensure adequate pattern complexity of restriction fragments and permit strain discrimination.

Pulsed-field gel electrophoresis is the most sensitive to changes caused by selective environmental pressures in the host and is most useful if a method at the extremity of sensitivity is required but is limited as a typing method by this very sensitivity.

Of particular interest is the family of ET clusters obtained by MLEE which indicate the close genetic relatedness of the clinical CF isolates, inclusive of even the archived ATCC reference strain from a bronchial washing. Although isolates in these clusters represent many different RTs by conventional *EcoRI* analysis (Table 2) (and more by a discriminatory extended ribotyping using three additional endonucleases [13b]), their close genetic relationship is apparent in MLEE linkage analysis. In addition, and perhaps just as significantly, 7 of 15 strains representative of nosocomial infections unrelated to CF formed a single, closely related family of ET8 and ET11 while other nosocomial isolates were generally distant from each other genetically. This analysis of strain relationships is not possible when conventional or even extended ribotyping is used as the discriminator. When dendrographic analysis was attempted using RT data, the resulting dendrogram appeared more as a lawn rake than as a tree, indicative of the lack of information in this method with which to analyze stable genetic relationships. MLEE, however, appears to be a robust technology ideally suited to analysis of genetic relatedness which ignores changes in genotype identified by ribotyping, which provides individual strain signatures suitable for identifying their patient origins rather than that of their family. Both are, of course, equally valuable in their place when the complete characterization of isolates is desirable.

In the cases of point source nosocomial outbreaks in this study, the isolates clustered in unique ETs and were typified by a single, stable RT per outbreak.

When the mutational evolution of a strain occurs in the same CF patient over years with little evidence for outside acquisition, we have observed in several cases that selective in vivo pressures may frequently alter the pulsed-field gel electrophoresis pattern and also the RT but that the ET does not change (13a). This is in contradiction to previous reports which have documented RT stability of serial pulmonary isolates (15, 18). Discrepant observations such as these need clarification by a definitive evaluation of archived strains from both pediatric and adult CF patients acutely and chronically infected with *P. cepacia*. Such studies will establish whether genomic variation of the same colonizing strain is occurring in vivo or whether the patients are suprainfected with multiple or distinct strains and perhaps, if these changes are associated with clinical deterioration, may identify strains of special virulence.

It seems apparent from these and other observations that the minor genomic base changes that alter the number and location of specific restriction recognition sequences and hence give rise to altered restriction fragment length polymorphism and RT patterns do not frequently result in amino acid alteration compatible with enzyme functions that are the source of allelic (electrophoretic) changes in MLEE. These changes are unselective of virulence or transmission characteristics, being initiated by background mutational events characteristic of bacterial DNA (27), but we suggest that the ET profile better describes strains that have been selected for virulence by the in vivo milieu of the CF patient. CF patients and those compromised by nosocomial experiences may also select the more virulent representatives from the general *P. cepacia* population of all possible ETs. MLEE analysis, because of its characteristics, will be especially useful in these circumstances for the investigation of pathogenesis, carrier state, and transmission.

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Production of Lipase by Clinical Isolates of *Pseudomonas cepacia*

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Ten clinical isolates of *Pseudomonas cepacia* from the sputum of cystic fibrosis patients were examined for the ability to produce lipase. Lipase substrates used included egg yolk agar, four different polyoxyethylene sorbitans (Tweens), and *p*-nitrophenylphosphorylcholine, a chromogenic substrate used to assay for phospholipase C. Lipase activity was detected in the filtrates of organisms grown to the exponential phase in either tryptose minimal medium or chemically defined medium. Lipase activity increased in the filtrates if the cultures were allowed to proceed into the stationary phase. None of the isolates produced phospholipase C. Lipase activity on Tween 20 ranged from 41.6×10^{-3} to 640.0×10^{-3} U/ μ g of protein. The activity was similar or slightly lower when Tween 40, 60, or 80 was used as the substrate. There was no correlation between lipase activity on Tween and that demonstrated on egg yolk agar. Lipase activity increased as pH increased from 7.0 to 9.0. Boiling for 5 min resulted in 66% loss of enzyme activity. The remaining activity continued to decrease with increasing boiling time. The enzyme was purified by gel filtration on Sephadex G-200, and the resultant preparation, when subjected to polyacrylamide gel electrophoresis, resulted in a single protein band (molecular weight, approximately 25,000) from which lipase activity could be eluted. The purified lipase was not cytotoxic to HeLa cells, nor was it toxic when injected intravenously into mice.

Pseudomonas cepacia, previously known by the synonyms *P. multivorans* and *P. kingae* (20), was characterized in 1950 by Burkholder (4) as the causative agent of bacterial rot in onion bulbs. Once thought only to be a phytopathogen, this organism is now recognized as an important opportunistic agent of human disease (19, 22). Recently, it has received a good deal of attention owing to its increasing association with fatal pulmonary infections in patients with cystic fibrosis (CF) (10, 25, 27). It is not closely related to *P. aeruginosa*, the most common organism isolated from the respiratory tract of CF patients (26), but, based on nucleic acid homology, is more closely related to the *P. pseudomallei* RNA group II (18).

Relatively little is known concerning the virulence factors of this organism. It has been shown by McKevitt and Woods (16) that *P. cepacia* produces a number of extracellular products including protease, gelatinase, hemolysin, and lipase. A role for any of these extracellular products in the disease produced by this organism has not been demonstrated. Also, these investigators were unable to demonstrate the production of exotoxin A or exoenzyme S by *P. cepacia* (16).

Although other extracellular bacterial enzymes such as proteases have received more attention in the study of possible virulence factors, there is evidence to indicate that lipases, particularly phospholipases, may play an important role in virulence. The alpha-toxin of *Clostridium perfringens* has been shown to be a lecithinase (15). *Staphylococcus aureus* produces a lipase which may, by hydrolyzing the lipids on the epithelial surface of human beings, enhance the colonization of the skin by this organism (29). Esselmann and Liu (8) reported that a number of gram-negative organisms, including *Vibrio cholerae* and several *Pseudomonas* species, also produce lipases, most notably phospholipase C. This enzyme is a lecithinase which catalyzes the hydro-

lysis of phosphatidylcholine, a phospholipid found in the membranes of animal cells, into phosphorylcholine and diacylglycerol (8). There is evidence that this enzyme is a virulence factor in pulmonary infections (13, 21). Liu (13) has suggested that the cytopathology to lung tissues in pulmonary infections by *P. aeruginosa* is due to the action of phospholipase C on phospholipids which make up the surfactant covering mammalian lung surfaces.

Since *P. cepacia* has often been described as being lipolytic (2, 5, 12, 16, 17, 23), we decided to investigate the incidence and nature of the lipase produced by this organism. Lipase activity of *P. cepacia* is well documented in the literature. Starr and Burkholder (23) described lipolytic activity by *Pseudomonas* species as early as 1941. In 1959, Morris and Roberts (17) isolated a group of pseudomonads from soils in Trinidad which they described as "strongly lipolytic." These isolates were later characterized as *P. cepacia* (2). McKevitt and Woods (16) reported that 32 of 48 strains of *P. cepacia* isolated from CF patients demonstrated lipase activity on egg yolk agar (16). Carson et al. (5) isolated three strains of *P. cepacia* from distilled water and assayed them for the ability to hydrolyze fat using synthetic substrates, Tweens 20, 40, and 80. All three strains were able to hydrolyze these substrates.

The first objective of this study was to establish the conditions which would lead to the best quantitation of lipase production by *P. cepacia*. Second, we were interested in quantitative examination of a number of clinical isolates of this organism for lipase production and whether any of these clinical isolates produced phospholipase C. Finally, we wished to purify the enzyme responsible for the hydrolysis of fat and examine its potential for toxicity in tissue culture and in vivo.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study were isolated from sputum samples from patients diagnosed as having CF. Strains 48b, 90ee, 99bb,

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155f, 224c, and 710m were obtained from J. D. Klinger, Rainbow Babies' and Children's Hospital, Cleveland, Ohio, and strains K19-2, K56-2, K30-6, and R5231-2 were obtained from C. L. Prober, The Hospital for Sick Children, Toronto, Ontario, Canada. All strains were maintained frozen in tryptic soy broth and 20% glycerol at -70°C . Liquid cultures were grown in Anwar chemically defined medium consisting of 3 mM KCl, 12 mM $(\text{NH}_4)_2\text{SO}_4$, 3.2 mM MgSO_4 , 1.2 mM K_2HPO_4 , 0.02 mM FeSO_4 , 3 mM NaCl, and 20 mM glucose in 50 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS; Sigma Chemical Co., St. Louis, Mo.), pH 7.6 (1). One-liter cultures were grown in 2-liter shaker flasks at 37°C in a water bath adjusted to 200 rpm. After 24 h of incubation, the cultures were harvested by centrifugation at $17,000 \times g$ for 30 min at 4°C . The supernatant was concentrated to dryness by lyophilization, reconstituted to 50 ml with 50 mM Tris hydrochloride (Sigma) (pH 7.6), and dialyzed for 2 days against the same buffer at 4°C with daily changes of buffer.

For the assay of phospholipase C activity, 2-ml cultures were grown in tryptose minimal medium (120 mM Tris hydrochloride buffer [pH 7.2], 0.1% tryptose [Difco Laboratories, Detroit, Mich.], 20 mM $(\text{NH}_4)_2\text{SO}_4$, 1.6 mM CaCl_2 , 10 mM KCl, 50 mM glucose) (24). Egg yolk agar plate assays were done on tryptic soy agar with the addition of 0.11% CaCl_2 and 5% egg yolk.

Assays. Isolates were screened for the ability to produce lecithinase activity on egg yolk agar by the method described by Esselmann and Liu (8). Lecithinase-positive colonies were identified by an opaque zone extending from the edge of the colony.

Lipase activity was also measured with polyoxyethylene sorbitans (Tweens 20, 40, 60, and 80; Sigma) as substrates by the method described by Tirunaryanan and Lundbeck (28). Tweens 20, 40, 60, and 80 are esters of lauric, palmitic, stearic, and oleic acids, respectively. Briefly, the reaction mixture consisted of 0.1 ml of 10% Tween in 50 mM Tris hydrochloride (pH 7.6) (Tris buffer), 0.1 ml of 1 M CaCl_2 in Tris buffer, 0.5 ml of concentrated culture supernatant, and 2.3 ml of Tris buffer. Duplicate samples were prepared for each isolate tested and incubated in a 37°C water bath for 2 h. Reagent blanks were prepared with 0.5 ml of deionized water instead of supernatant. Tween is cleaved to produce a fatty acid and an alcohol. In the presence of calcium, an insoluble fatty acid salt is formed, giving a precipitate which can be measured turbidimetrically at 400 nm. One unit of lipase activity was defined as that amount of enzyme which, after 2 h under the conditions of the assay, resulted in an increase of optical density at 400 nm (OD_{400}) of 0.01. Activity was reported as units per microgram of total protein.

p-Nitrophenylphosphorylcholine (Sigma) was used to assay for the production of phospholipase C by the method of Kurioka and Matsuda (11). Phospholipase C hydrolyzes *p*-nitrophenylphosphorylcholine to phosphorylcholine and *p*-nitrophenol in the presence of sorbitol or glycerol and Zn^{2+} . *p*-Nitrophenol is chromogenic and was measured at 410 nm. Phospholipase C (type 1; Sigma) from *C. perfringens* was used as a positive control. The amount of total protein in the samples was quantified by the method of Lowry et al. (14).

Effects of boiling on lipase activity. Four sets of duplicate samples of concentrated supernatant from strain 90ee were placed in a boiling-water bath for 5, 10, 15, and 30 min, respectively. After boiling, the samples were cooled and assayed for lipase activity on Tween 20 in 50 mM Tris buffer, pH 7.6. Activity, reported as units per microgram of total

protein, was compared with that of the unheated control, and percent activity was calculated.

Growth curve experiments. For growth curve experiments, 1 liter of Anwar defined medium was inoculated with washed cells to an OD_{540} of 0.15. Optical density was determined at 540 nm at 1-h intervals. Samples (10 ml) were then transferred to centrifuge tubes, and the cells were harvested by centrifugation for 30 min at $17,000 \times g$. The supernatants were frozen, lyophilized, reconstituted to 1.0 ml with 50 mM Tris hydrochloride (pH 7.6), and dialyzed against the same buffer. Duplicate samples were then assayed for lipase activity and total protein.

Gel filtration chromatography. The concentrated supernatant from five 1-liter 24-h-old cultures of 90ee was lyophilized to dryness, dialyzed, and suspended in 5.0 ml of Tris buffer (50 mM, pH 7.6). This concentrate, referred to as stage 1 enzyme, was applied to a Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) gel filtration column (90 by 2.5 cm) which had been equilibrated overnight at 4°C with 50 mM Tris buffer, pH 7.6. Fractions were monitored for A_{280} by a flowthrough 2138 Uvicord S monitor (LKB Instruments, Inc., Rockville, Md.) and recorded by a 6520-5 Chopper Bar six-channel recorder (LKB Instruments). A total of 145 fractions of 4.6 ml each were collected, and every fifth fraction was assayed for lipase activity. Those fractions which demonstrated lipase activity were pooled and lyophilized to dryness. After dialysis against 50 mM Tris buffer (pH 7.6), this preparation was referred to as stage 2 enzyme.

PAGE. The lyophilized sample consisting of the pooled fraction from the Sephadex G-200 column was reconstituted in 2.0 ml of deionized water and dialyzed overnight against 50 mM Tris (pH 7.6) at 4°C . The sample was then further concentrated by lyophilization and reconstituted in 250 μl of deionized water. The sample was applied to analytical polyacrylamide gels and analyzed by the Davis (6) procedure for polyacrylamide gel electrophoresis (PAGE).

Electrophoresis was done at 2 mA per gel until the tracking dye passed through the stacking gel and at 5 mA thereafter until the dye was approximately 1 cm from the bottom of the gel. Gels were then removed from the tubes and stained for 2 h at room temperature in 0.2% Coomassie blue in 5:1:5 (vol/vol/vol) methanol-acetic acid-water. Destaining was accomplished in 5% methanol-7.5% acetic acid at room temperature. The stained gels were placed in a test tube (13 by 100 mm) and scanned at 580 nm on a Beckman Du70 spectrophotometer. The unstained gels were cut into 5-mm sections. Each section was put into a dialysis bag with 1 ml of Tris buffer (50 mM, pH 7.6), mashed, and dialyzed against the same buffer at 4°C for 72 h. After dialysis, the contents of each bag were analyzed for lipase in an attempt to locate where in the gel the activity resided.

Effects of pH on lipase activity. Five sets of duplicate samples of stage 2 enzyme from strain 90ee were assayed for lipase activity on Tween 20 as described above. The pH of the 50 mM Tris buffer used in the assay was adjusted with 1 N NaOH to 7.0, 7.5, 8.0, 8.5, or 9.0. Activity was reported as units per microgram of total protein.

Lecithinase activity of purified enzyme. Stage 2 enzyme was used to fill a 2-mm well cut into an egg yolk agar plate. The plate was incubated at 37°C for 6 h and inspected for a zone of opacity around the well. Phospholipase C (10 $\mu\text{g}/\mu\text{l}$) from *C. perfringens* (Sigma) was used as a positive control.

Cytotoxicity assay. HeLa cells were grown to monolayers in microtiter wells at 37°C under 5% CO_2 in Eagle minimal essential medium (Whittaker-M. A. Bioproducts, Walkers-

ville, Md.) supplemented with 10% newborn calf serum, 64 μg of penicillin per ml, and 100 μg of streptomycin per ml. Twofold serial dilutions of the stage 2 preparation containing 30 μg of protein and having a specific activity of $2,127.9 \times 10^{-3}$ U/ μg of total protein were made in Tris buffer (50 mM, pH 7.6). A 50- μl sample of each dilution was added to the appropriate microtiter well containing HeLa cells. Purified toxin B from *Clostridium difficile* was used as a positive control (7). Supernatant from a nontoxicogenic culture of *C. difficile* served as a negative control. The plates were incubated for 24 h at 37°C under 5% CO₂ and then examined microscopically. Cytotoxicity was scored as any rounding or actinomorphous change in the cells.

Animal studies. Five 20-g female Swiss Webster mice (Cox Laboratories, Indianapolis, Ind.) were injected intravenously with 0.1 ml of the stage 2 lipase preparation containing 30 μg of protein and having a specific activity of $2,127.9 \times 10^{-3}$ U/ μg of total protein. Controls were injected with 0.1 ml of Tris buffer (50 mM, pH 7.6). The animals were observed for 72 h.

RESULTS

Activity of *P. cepacia* lipase on a variety of substrates. Ten clinical isolates of *P. cepacia* from the sputum of CF patients were examined for their ability to produce lipase activity on a number of different substrates, including egg yolk agar, *p*-nitrophenylphosphorylcholine, and four different Tweens. The egg yolk reaction is commonly used to detect the activity of lecithinase, which splits lecithin (phosphatidylcholine), liberating phosphorus and choline, usually with the deposition of fat (15). Of the 10 isolates tested (48b, 224c, 710m, K19-2, K56-2, and R5231-2), 6 produced lecithinase.

We wanted to determine whether the lipase activity of *P. cepacia* was due to phospholipase C. We assayed the concentrated culture supernatant from each of the 10 isolates and were unable to detect any phospholipase C activity on *p*-nitrophenylphosphorylcholine. The control, phospholipase C, type 1, from *C. perfringens*, gave a strong positive reaction with an OD₄₀₅ of 0.250 for a preparation of 0.1- $\mu\text{g}/\text{ml}$ concentration.

The Tween assay permitted us to quantitate the lipase activity. Lipase activity was expressed as units of activity per microgram of total protein. The activity reported in this manner for Tween 20 ranged from 41.6×10^{-3} to $640.0 \times$

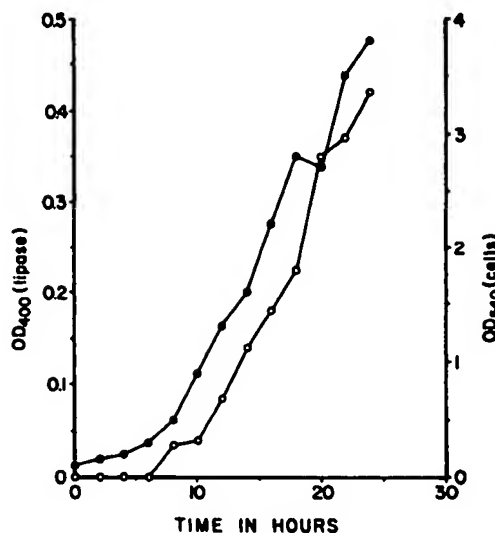


FIG. 1. Effect of growth phase (●) on lipase activity (○) produced by *P. cepacia* 90ee. Cells were grown in Anwar minimal defined medium. At 2-h intervals, 10-ml samples were taken from the culture and duplicate samples of the concentrated supernatant were assayed for lipase on Tween 20 as described in Materials and Methods. The mean values were plotted as a function of time. The growth of the organism was determined spectrophotometrically at OD₅₄₀, and the lipase assay was read at OD₄₀₀.

10^{-3} U/ μg of protein (Table 1). There was no apparent correlation between the activity detected on egg yolk agar and that demonstrated on Tween. For example, strain 90ee consistently produced the most lipase activity (640.0×10^{-3} U/ μg of protein) against Tween 20 but was negative on the egg yolk agar assay, and strain K56-2, the organism which produced the least lipase activity (41.6×10^{-3} U/ μg of protein) against Tween 20, was positive in the egg yolk agar assay.

We also used Tweens 40, 60, and 80 to assay for lipase activity produced by all 10 isolates (Table 1). All strains gave a measurable reaction on Tween 20. Similar values were observed with Tween 40 with the exceptions of strain 710m, which showed greater activity against Tween 40, and strain K56-2, which showed none at all. Activity was somewhat less on Tween 60 and was least on Tween 80. In view of these results, we elected to use Tween 20 as a substrate for assay in subsequent experiments.

Relationship between *P. cepacia* growth cycle and production of lipase. Because strain 90ee produced relatively large amounts of lipase per microgram of protein on Tween 20, we elected to use this strain to determine at what point in the growth curve maximum production of lipase occurred (Fig. 1). This strain had a doubling time in minimal medium of about 2 h, and the growth curve was biphasic. Lipase activity began to appear at the beginning of the log phase (6 h), and its increase closely paralleled the increase in the *P. cepacia* population until the end of the first log phase (18 h), when the growth curve leveled off before entering a second phase of growth at 20 h. Lipase activity, however, continued to accumulate as the cells passed through the first stationary phase and into the second log phase of the curve. Strain K19-2 likewise continued to produce lipase when the culture was allowed to proceed into the stationary phase (data not shown). Although growth experiments were conducted in

TABLE 1. Lipase activity of 10 clinical isolates of *P. cepacia* on four different tweens

Strain	Activity ^a on the following substrate:			
	Tween 20	Tween 40	Tween 60	Tween 80
48b	127.3 \pm 1.2	128.2 \pm 5.8	117.0 \pm 0.7	103.3 \pm 0.9
90ee	640.0 \pm 0.3	650.4 \pm 43.7	473.3 \pm 0.0	413.4 \pm 23.0
99bb	131.2 \pm 0.9	142.5 \pm 6.6	124.3 \pm 7.7	95.4 \pm 5.7
155f	329.7 \pm 1.4	297.1 \pm 4.8	291.3 \pm 10.0	211.0 \pm 25.8
224c	635.0 \pm 21.0	640.0 \pm 0.0	360.0 \pm 78.0	379.0 \pm 59.0
710m	46.6 \pm 2.0	92.6 \pm 9.2	86.6 \pm 3.2	53.9 \pm 12.8
K19-2	81.7 \pm 12.7	62.3 \pm 9.6	64.1 \pm 1.2	58.8 \pm 3.6
K56-2	41.6 \pm 1.6	ND ^b	60.0 \pm 4.6	30.0 \pm 0.8
K30-6	427.8 \pm 0.0	420.4 \pm 7.0	337.5 \pm 5.5	271.3 \pm 7.0
R5231-2	304.7 \pm 8.0	308.8 \pm 0.2	174.1 \pm 11.2	160.4 \pm 30.2

^a All activities are expressed as 10^{-3} units of lipase activity per microgram of total protein \pm standard error of the mean of duplicate samples.

^b ND, None detected.

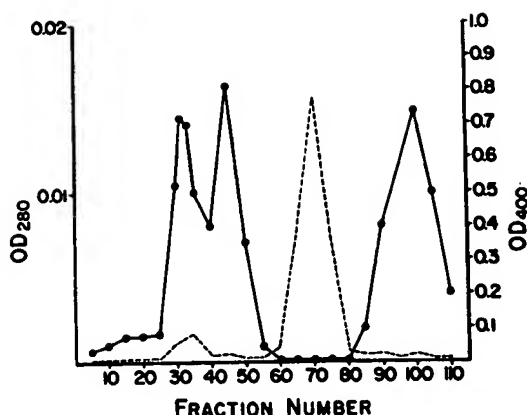


FIG. 2. Elution profile of concentrated 90ee culture supernatants (5 liters/24-h cultures) from Sephadex G-200 gel filtration column. The eluate was continuously monitored for protein at 280 nm (—), and every fifth tube was assayed for lipase activity on Tween 20 (---).

Anwar defined medium, similar lipase activity was also noted when cultures were grown in tryptose minimal medium (data not shown).

Effects of boiling lipase activity. Heating in boiling water for 5 min reduced the lipase activity on Tween 20 by 66%. The activity could be further reduced by continued boiling. Boiling for 10 min resulted in a loss in 71% of lipase activity, 15 min of boiling destroyed 78% of the activity, and boiling for 30 min resulted in a loss of 83% of the activity shown by the unheated control.

Gel filtration and PAGE. Gel filtration chromatography of concentrated culture supernatants on Sephadex G-200 resulted in the elution profile shown in Fig. 2. Lipase activity was eluted in fractions 60 to 80. This elution volume is approximately the same as that of chymotrypsinogen (molecular weight, 25,000). This peak of activity does not correspond to any major protein peak as measured by OD₂₈₀. However, when these fractions were pooled and subjected to PAGE, a single band appeared. The 80-mm Coomassie blue-stained gels were placed in a test tube (13 by 100 mm) for scanning at 580 nm. Figure 3 shows that the tubes were scanned from 20 to 90 mm. The first 7 mm of the scan showed the densely stained stacking gel. A diffusely stained area continued approximately 10 mm into the running gel. We believe this to be artifactual, as it appeared in all the gels of this run, including the blank. The peak at 37 mm represented a single band which corresponded to a single peak of lipase activity eluted from the corresponding location in unstained gels. The dense region near the end of the scan is the tracking dye front, and the distortion of the light beam is due to the shape of the end of the tube.

Effect of pH on lipase activity. Lipase activity of stage 2 enzyme on Tween 20 increased as the pH increased in increments of 0.5 from 189.9×10^{-3} U/ μ g of total protein at pH 7.0 to 404.6×10^{-3} U/ μ g of total protein at pH 9.0 (Table 2).

Cytotoxicity and animal studies. There was no detectable cytotoxicity to HeLa cells caused by stage 2 preparations of this enzyme in the concentrations we examined. There was, however, noticeable rounding the cells subjected to *C. difficile* toxin B. There were no deaths or morbidity among the five mice injected intravenously with stage 2 lipase.

Lecithinase activity of stage 2 lipase. The lipase which hydrolyzes Tween 20 showed no discernable reaction when

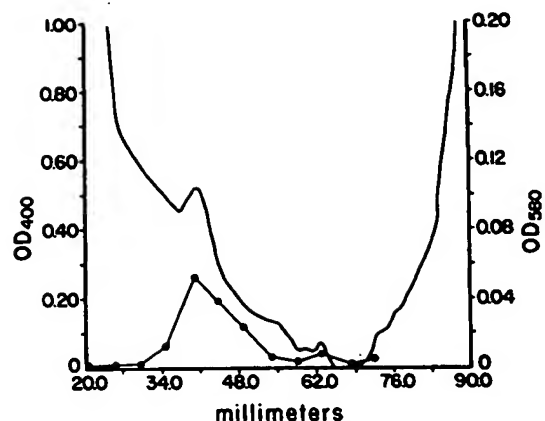


FIG. 3. Profile of densitometric scan (—) and eluted lipase activity (●—●) from polyacrylamide gels of stage 2 enzyme from 90ee culture supernatants. Stained gels were scanned at 580 nm. Unstained gels were eluted and assayed for lipase activity on Tween 20.

applied as stage 2 enzyme to egg yolk agar. Phospholipase C ($10 \mu\text{g}/\mu\text{l}$) resulted in a zone of opacity approximately 1 cm in diameter.

DISCUSSION

Many microorganisms elaborate enzymes which are lipolytic. Methods for screening for these enzymes include techniques which measure a visible, physical change in the substrate, such as the egg yolk agar method (8) and the spirit blue agar method of Starr and Burkholder (23). However, both of these methods involve agar plates and are somewhat time consuming and cumbersome. Other methods assay for reaction products, such as the alcohol released or the freed fatty acids. The Tween assay offered a fast, convenient technique for assaying for fatty acids. It was described and used by Tirunarayanan and Lundbeck (28) to assay for lipase produced by staphylococci. We found the procedure useful for rapid screening of large numbers of *P. cepacia* isolates for lipase production.

Macfarlane et al. (15) characterized the nature of the egg yolk reaction produced by *C. perfringens* as the activity of a lecithinase. However, egg yolk is a mixture of lipids, phospholipids, lipoproteins, and other complex components. Therefore, the egg yolk reaction may be caused by any of a number of enzymes acting on a variety of substrates. We showed that the activity produced by these 10 clinical isolates on egg yolk agar is apparently uncorrelated with that produced on any of the Tweens. It seems likely that these

TABLE 2. Effect of pH on lipase activity of *P. cepacia* 90ee

pH	10^{-3} U/ μ g of total protein ^a
7.0	189.5 ± 14.1
7.5	324.0 ± 3.6
8.0	361.7 ± 17.7
8.5	387.3 ± 9.4
9.0	404.7 ± 6.4

^a 10^{-3} Units per microgram of total protein \pm standard error of the mean of duplicate samples. Assay was done on Tween 20 in 50 mM Tris buffer at designated pH.

reactions are due to different enzymes. This is supported by the fact that purified preparations of the enzyme which hydrolyzed Tween 20 gave a negative reaction on egg yolk agar. Furthermore, since no phospholipase C was detected, it appears that the egg yolk agar reaction described here is due to some enzyme other than a lecithinase.

The Tween assay also provided a choice of substrates. Tirunarayanan and Lundbeck (28) reported that the amount of measurable activity of staphylococcal lipase varied depending on which Tween was used as a substrate. Horlein and Pilz (9) reported that human serum protein lipase activity was twice as great on Tween 80 as on Tween 60. The variability of the reactions on the various Twens may be a matter of how well the enzyme fits the synthetic substrate. Twens 20, 40, 60, and 80 are esters of lauric, palmitic, stearic, and oleic acids and have 12, 16, 18, and 18 carbons, respectively, in their chains. Oleate differs in that it also has a double bond between carbons 9 and 10. Tween 80 was least hydrolyzed by these organisms. Tween 20 was hydrolyzed by all strains. However, some of the strains, 710m for instance, were better able to use Tween 40 or Tween 60 than Tween 20. This suggests that the lipase which most effectively hydrolyzes Tween varies from strain to strain.

The biphasic nature of the growth curve of strain 90ee (Fig. 1) is a reproducible phenomenon. We repeated this experiment three times and it was always present. Dilution plate counts showed the second phase to be due to an increase in the numbers of viable organisms. These experiments were conducted in minimal medium, and it is possible that the organisms were exhausting the medium of glucose and converting to an alternative carbon source at this point. *P. cepacia*, which is able to use a variety of carbon sources, is known to synthesize and accumulate large intracellular stores of poly- β -hydroxybutyric acid (17). This could conceivably be used as an energy source in the absence of glucose.

Lipase activity continued to develop in all the culture supernatants when the cells were allowed to enter into the stationary phase, indicating that the enzyme is stable under culture conditions and not subject to proteolytic digestion by any of the proteases produced by the organism (16). Furthermore, during the course of these experiments, we observed little loss of activity during manipulation such as freezing and thawing, lyophilization, and dialysis.

When the enzyme was heated in a boiling-water bath for 5 min, two-thirds of its activity was lost. Upon continued heating, activity continued to be gradually and steadily lost. This again indicates the possibility of more than one enzyme. An alternative explanation would be more than one species of the same enzyme, one form being complexed or aggregated in such a way that makes it very resistant to denaturation by heating. Boiling for 30 min resulted in only 83% reduction of activity.

Lipase activity on Tween 20 increased as pH was increased from 7.0 to 9.0. The greatest increase in activity occurred between pH 7.0 and 7.5, after which the increase became more gradual (Table 2). We believe the optimum pH to be well into the alkaline range, although the constraints of the Tween assay prevented us from measuring activity above pH 9.0 or below pH 7.0. Above pH 9.0, a dense white turbidity forms when CaCl_2 is added to the Tris buffer, interfering with the assay. In addition, pH 7.0 is near the lower end of the buffering capacity of Tris (pK 8.2), and the reaction may be self-limited by the acid evolved.

Gel filtration chromatography of concentrated supernatants resulted in three major peaks which absorb at 280 nm

(Fig. 2). However, practically all the lipase activity eluted from the column was contained in a pool midway between the second and third peaks, indicating a molecular weight of approximately 25,000. It appeared that the lipase was being eluted from the column relatively free of other proteins. To test this hypothesis, we pooled the lipase-containing fractions, concentrated them, and subjected them to PAGE. This resulted in a single protein band. When unstained gels were cut into sections and eluted, the section corresponding to this band contained the majority of the lipase activity eluted from the gel (Fig. 3).

This physiological role of extracellular lipases produced by bacteria is probably nutritional. Some may hydrolyze exogenous triglycerides to provide free fatty acids for use as an energy source. Phospholipase C produced by *P. aeruginosa* is likely a phosphate-scavenging mechanism. Its production is suppressed by the addition of inorganic phosphate (3). Many lipases are produced constitutively, although their production may be influenced by the nutritional and physical conditions of the culture (11). The lipase of *P. cepacia* is produced both in defined medium and in tryptose medium. The fact that it is produced in the absence of exogenously supplied substrate plus the fact that its production correlates with the growth curve of the organism suggests that it is constitutive.

Colonization of CF patients by *P. cepacia* may be asymptomatic or may result in chronic respiratory disease. However, some patients develop fulminant, necrotizing pneumonia which is progressive and rapidly fatal (10, 25, 27). There is often extensive destruction of lung parenchyma (27), suggesting that some toxin or enzyme is acting directly on pulmonary tissue. Purified lipase from 90ee was not found to be cytotoxic for HeLa cells. This finding is in concurrence with those of McKevitt and Woods (16), who reported that culture supernatants from *P. cepacia* were not cytotoxic to any of the tissue culture cell lines which they examined. In addition, we noted no ill effects in mice intravenously injected with 30 μg of the purified lipase. However, all the strains examined thus far in this laboratory are clinical isolates from CF patients and all of them produce lipase. Purification of this lipase has made possible further experiments which should yield information concerning the possible role of this enzyme in human pulmonary disease.

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Invasive aspergillosis presenting as pericarditis and cardiac tamponade

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A M E R I C A N C O L L E G E O F



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occurred in association with probable pneumoconiosis: Carasso et al³ reported a 48-year-old woman with a bronchoesophageal fistula due to silicotic mediastinal lymphadenopathy, and Frew⁴ reported a 51-year-old man with a chest roentgenogram suggestive of pneumoconiosis and dysphagia due to a tuberculoma in the esophagus. Mediastinal lymph node enlargement due to other granulomatous diseases such as sarcoidosis and histoplasmosis have been shown to compress the esophagus.¹⁻²

Although this patient had no specific signs or symptoms of tuberculosis, tissue cultures of the cervical lymph nodes grew *M intracellulare*. The increased incidence of *Mycobacterium tuberculosis* in silicotic patients is well known. Also atypical mycobacterial infections appear to be more frequent in patients with pneumoconiosis.⁵⁻⁶ Blacks generally have a lower incidence of clinical atypical mycobacteriosis than whites, but our patient's silicosis and diabetes mellitus may have increased his risk to infection.⁵ His anemia of chronic disorders may be related to the mycobacteriosis.⁷

Although this patient was lost to follow up by the chest service, it is interesting to speculate what the most appropriate course of therapy for him would have been. We recommended that he undergo a thoracotomy to remove the mediastinal lymph nodes compressing the esophagus and perhaps reduce the burden of tuberculous tissue as well. We also would have administered chemotherapy with five to six antituberculosis drugs because *M intracellulare* is notoriously resistant to conventional therapy.⁸ Atypical mycobacterioses are even more difficult to treat in patients with pneumoconiosis.⁶

This is the first reported case, to our knowledge, in which silicotic lymphadenopathy complicated by *M intracellulare* infection produced dysphagia by extrinsic compression of the esophagus. Silicosis should be considered among other granulomatous diseases such as mediastinal granuloma, tuberculosis, and sarcoidosis in the differential diagnosis of dysphagia.

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Invasive Aspergillosis Presenting as Pericarditis and Cardiac Tamponade*

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A 38-year-old leukemic patient developed pericarditis and cardiac tamponade due to *Aspergillus niger* one month after undergoing bone marrow transplantation. She failed to improve even though amphotericin B and rifampin therapy had been initiated before infection was evident. Her unique case illustrates both the unusual presentations of invasive aspergillosis and the difficulty of diagnosing and treating this increasingly common disease

Invasive aspergillosis is recognized with increased frequency among immunocompromised patients.¹⁻³ However, recognition often is delayed by the unusual and nonspecific manifestations of this condition. We present the case of a bone marrow transplant recipient in whom pericarditis and cardiac tamponade were the first clinical indications of invasive aspergillosis.

CASE REPORT

The patient was a 38-year-old woman with acute myelomonocytic leukemia who was transferred to the Fred Hutchinson Cancer Research Center (FHCRC; UPN907) in second relapse. Previous chemotherapy had included cytarabine, 6-thioguanine, and daunomycin. Admission laboratory findings included pancytopenia with a peripheral neutrophil count of 150/cu mm. Chest roentgenogram and ECG were normal (Fig 1, left). The patient was placed in laminar air flow and was given prophylactic oral antibiotics. She was prepared for transplantation with 1 mg/kg/body weight of nitrogen mustard followed by 1,200 rads of total body irradiation in six divided doses, as per FHCRC protocol.⁴

During preparation, the patient was given intravenous carbenicillin and gentamicin for fever. Intravenous amphotericin B, 25 mg/day, and rifampin, 300 mg/day, were added when blood cultures grew *Candida tropicalis*. Two weeks after admission, the patient received a bone marrow transplant from her HLA-matched sibling, who also was the source of daily granulocyte infusions.

The patient's posttransplantation course was complicated by an episode of pulmonary edema which was thought to be due to pre-existent anthracycline cardiac toxicity and fluid overload. Her respiratory status improved with diuretics, and her chest roentgenogram cleared but for a small right middle lobe (RML) infiltrate. She then developed toxic enteritis which was treated with intravenously administered corticosteroids.

Three weeks after admission, the patient noted anterior chest pain which radiated into her throat and was intensified in the supine position. The pain was felt to be due to *Candida* esophagitis after barium swallow disclosed esopha-

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FIGURE 1. Chest roentgenograms taken on admission (1A, left), at the time pericarditis became evident (1B, center), and shortly before death (1C, right). Note progressive increase in RML infiltrate and cardiac silhouette caused by invasive aspergillosis.

geal ulcerations. The pain worsened over the next two days, and the patient developed a rapid and irregular pulse. A pericardial friction rub then was noted, and chest roentgenogram revealed that the RML infiltrate had increased and now was associated with an enlarged cardiac silhouette (Fig. 1, right). An ECG showed low voltage and atrial fibrillation, and echocardiogram demonstrated a pericardial effusion of approximately 50 ml.

The patient was taken to surgery, where the right side of the chest was entered through a parasternal incision. The RML was found to be firm and pale, the pericardium was covered with a shaggy exudate, and the pericardial sac contained 50 ml of serosanguinous fluid. A pericardial window was created, and the pericardium and RML were biopsied.

On microscopic examination, the RML tissue was hemorrhagic and infarcted. Branching, septate hyphae were seen on the pleural surface; sheets of the same hyphae were found adjacent to and invading the pericardium. Cultures of the pericardium and pericardial fluid subsequently grew *Aspergillus niger*.

Amphotericin B was continued to a total dose of 750 mg, and the patient's neutrophil count rose to 700/cu mm, indicating increased graft function. The patient was stable for two days until her cardiac silhouette enlarged even more on chest roentgenogram, and she developed a 30-mm paradoxical pulse (Fig 1, center). That night, the patient required endotracheal intubation and mechanical ventilation for worsening cardiopulmonary function. Pericardiectomy was planned, but she developed massive hemoptysis and died in ventricular fibrillation the following day.

At autopsy, the patient's pericardium, lungs, pleurae, heart, kidneys intestines and thyroid gland were found to be involved with *Aspergillus*. The fungus had invaded the aorta and the left main and left anterior descending coronary arteries, as well as the proximal right pulmonary arterial tree.

DISCUSSION

Invasive aspergillosis is second only to candidiasis of the fatal mycoses seen in cancer patients.³ Although it occurs most commonly in persons with neoplasms, aspergillosis also has been reported in immunosuppressed persons with sarcoidosis and collagen vascular diseases

and in individuals undergoing renal, cardiac, and bone marrow transplantation.¹⁻³ Factors predisposing to aspergillosis include granulocytopenia, broad spectrum antibiotic therapy, and the administration of corticosteroids.^{1,2} All of these factors were present in this patient.

Aspergillus characteristically enters the body through the respiratory tract. In the lungs, tissue invasion usually is manifested by bronchopneumonia or by a distinctive pattern of hemorrhagic infarction. Such infarction, which was seen in this patient, occurs secondary to vascular invasion by mycelial elements with thrombosis and occlusion of the pulmonary vessels. The fungus then may spread from the lungs by direct invasion, or more commonly, by hematologic dissemination.²

The organs most often involved in disseminated aspergillosis are the lungs, intestine, brain, kidneys, liver, esophagus, and heart, in that order. Pericardial involvement is unusual, occurring in only three of 93 patients in a series from the Memorial Sloan-Kettering Cancer Center and four of 98 patients from the National Cancer Institute (NCI).^{1,2} None of the NCI patients had signs or symptoms suggestive of pericarditis or pericardial tamponade.² In 1962, Fraumeni and Fear⁵ described a lymphoma patient with distended neck veins and pulsus paradoxus, but that is the only reported case in which *Aspergillus* caused an obvious pericarditis.

Even when the diagnosis of aspergillosis is made, treatment often is unsuccessful. Therapy includes the administration of amphotericin B, flucytosine, and aerosolized nystatin, either alone or in combination.^{3,6,7} Rifampin and amphotericin B also have been used together.⁸

According to one recent report, the clinical outcome of cancer patients with aspergillosis correlates best not with the total dose of amphotericin but with the recovery of circulating neutrophils.^{1,3} Outcome also has been reported to improve with early diagnosis and treatment.⁹ However, this patient already was receiving

amphotericin B and rifampin when her aspergillosis became apparent, and she had achieved partial bone marrow engraftment. Her case underscores the difficulty of diagnosing and treating invasive aspergillosis in immunocompromised hosts as well as the unusual manifestations of this disease.

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Spontaneous Return of Patency in a Completely Occluded Coronary Artery*

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We observed a 56-year-old man in whom an occluded right coronary artery was observed to be widely patent on a subsequent angiogram 40 months later. This "regression," which occurred without a change in his risk factors, shows that manipulation of risk factors can be proven to be a cause of regression only in controlled studies.

Serial coronary arteriography has shown that the expected course of coronary atherosclerosis is a steady and relentless progression of disease.¹⁻⁴ The rate of progression of an individual lesion is highly unpredictable but there appears to be a relationship between the number and severity of risk factors such as cigarette smoking, hypertension, and hyperlipoproteinemia and the rate of progression of coronary artery disease.^{1,5}

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Regression of atherosclerosis in animals^{6,7} and in the peripheral vessels in man has been shown in response to manipulation of the atherogenic stimuli.^{8,9} Because it is generally presumed that coronary artery disease always progresses, or that if regression is possible it occurs only after vigorous therapy of atherogenic stimuli, we are reporting a patient with a prior inferior myocardial infarction in whom angiographic "improvement" of a right coronary artery occlusion was demonstrated. The improvement occurred without treatment of known risk factors for coronary artery disease.

CASE REPORT

The patient is a 56-year-old man who presented to another hospital with acute chest pain, diaphoresis, and syncope in December 1974. The ECG showed evidence of an acute inferior myocardial infarction and complete heart block. He was treated with intravenous isoproterenol, atropine, and oral prednisone. Eight days after myocardial infarction, he had a right femoral artery embolus which was uneventfully removed.

After discharge from the hospital, he noted persistent chest pain and dyspnea on exertion. He was admitted for reevaluation in April 1975.

He was 177.8 cm (70 inches) tall and weighed 72.3 kg (160 pounds). The arterial blood pressure was 115/80 mm Hg. The fasting blood sugar level was 90 mg/100 ml. A plasma cholesterol determination was not made.

Coronary arteriography was performed using the Judkins femoral percutaneous technique. Cineangiograms were made of the right and left coronary arteries in the right anterior oblique and left anterior oblique projections. Large film serial roentgenograms using a rapid film changer programmed for ten exposures in three seconds were made of each coronary artery in the lateral, right anterior oblique, and left anterior oblique projections. Secobarbital, 100 mg intramuscularly, was given prior to the procedure. Nitroglycerin 0.4 mg sublingual, was given prior to introduction of the right coronary artery catheter. The procedure was accomplished without complication; there was no angina during the procedure.

The examination revealed a complete occlusion in the midportion of the right coronary artery, complete occlusion of the left anterior descending coronary artery, and irregularity without significant obstruction in the proximal left circumflex coronary artery (Fig 1). There was complete morphologic consistency of all films. The left ventriculogram demonstrated inferior and apical akinesis and an ejection fraction of 0.15. Coronary bypass surgery was not recommended because of the poor ventricular function and because distal coronary arteries suitable for bypass grafting were not identified.

He was treated with progressively larger doses of propranolol until the dose of that medication reached 320 mg per day. Digoxin, 0.25 mg, was given daily and a thiazide diuretic was given for a brief period. No other drug therapy was used.

When the patient was first evaluated at our hospital in April 1978, he continued to complain of severe angina and dyspnea on exertion. In the interim, he had continued to smoke 1½ packs of cigarettes per day as he had done for the previous 30 years. He had not changed his diet. His disability was such that he engaged in minimal physical activity.

He weighed 168 pounds. His arterial blood pressure was 130/85 mm Hg. He had bibasilar rales, an S3, and pedal

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Epidemiology of *Burkholderia cepacia* Infection in Patients with Cystic Fibrosis: Analysis by Randomly Amplified Polymorphic DNA Fingerprinting

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We fingerprinted a collection of 627 *Burkholderia cepacia* isolates from 255 patients with cystic fibrosis (CF) and 43 patients without CF and from the environment, by a PCR-based randomly amplified polymorphic DNA (RAPD) method with primers selected for their ability to produce discriminatory polymorphisms. The RAPD typing method was found to be reproducible and discriminatory, more sensitive than PCR ribotyping, and able to group epidemiologically related *B. cepacia* strains previously typed by both pulsed-field gel electrophoresis and conventional ribotyping. Seven strain types infecting multiple CF patients were found at several different CF treatment centers in Canada, the United States, the United Kingdom, France, and Australia, indicating the presence of epidemic strain types. Most CF patients were each colonized with a single strain type, and several patients harbored the same strain type for 5 or more years. *B. cepacia* isolates recovered from other clinical sources (44 isolates examined) and from the environment (58 isolates examined) possessed RAPD fingerprints that were generally distinct from CF-associated strain types (525 isolates examined). RAPD is a versatile fingerprinting method for studying the epidemiology of *B. cepacia*.

Cystic fibrosis (CF) is the most common potentially fatal autosomal recessive disease in North America, afflicting approximately 1 in 2,000 live births among Caucasians (5). Although *Pseudomonas aeruginosa* is the predominant respiratory pathogen in patients with CF (26), *Burkholderia* (formerly *Pseudomonas*) *cepacia* has emerged as a particularly problematic pulmonary pathogen in these patients. The organism is highly virulent in certain patients with CF (8, 30). It is intrinsically resistant to a wide range of antimicrobial agents (22), and there is considerable evidence that *B. cepacia*, unlike *P. aeruginosa*, can spread from one CF patient to another both within and outside the hospital (2, 7, 9, 13, 15). However, investigations finding no evidence of patient-to-patient transmission (27) or documenting only the transmission of one epidemic clone (29) suggest that the transmissibility of *B. cepacia* may vary depending on a number of factors including strain, treatment center, and CF patient population. It has become critically important to determine the risk factors for patient-to-patient spread of *B. cepacia* and to identify strains that are prevalent and pose the greatest risk of infection at CF treatment centers.

Several techniques have been employed for typing *B. cepacia*. Phenotypic methods such as serology, biochemical profile, or pigment production have been widely used (23) but are subject to instability because the phenotype of *B. cepacia* CF isolates may vary markedly (12). Multilocus enzyme electrophoresis, a phenotypic method, has also been applied to *B. cepacia* (9); multilocus enzyme electrophoresis demonstrated that CF isolates are clustered clonally and are generally distinct from nosocomial and environmental isolates; however, the

number of strains examined from non-CF sources was small. Genetic typing methods, such as ribotyping (28), have been shown to provide good specificity and sensitivity for epidemiological study of *B. cepacia* (23). Ribotyping analysis has been used to demonstrate patient-to-patient transmission (7, 13, 25), clustering of *B. cepacia* types at treatment centers (15), and transatlantic spread of one transmissible lineage (9, 29). Typing by pulsed-field gel electrophoresis (PFGE) has also demonstrated the spread of certain strains among CF patients (7, 25, 29); however, Steinbach et al. (27) reported no PFGE typing evidence for person-to-person transmission of *B. cepacia* at one CF treatment center. Three PCR-based methods for typing *B. cepacia* have also been reported: PCR ribotyping (11), randomly amplified polymorphic DNA (AP-PCR or RAPD) (2), and enterobacterial repetitive intergenic consensus sequence PCR (17).

Over the last 10 years, our laboratory has established a large collection of *B. cepacia* isolates from pediatric and adult CF treatment centers in Vancouver, British Columbia, Canada. This collection has recently been expanded to include isolates from other CF centers in Canada, the United States, the United Kingdom, France, and Australia, as well as many isolates from other clinical sources and from the environment. We sought to type this collection of isolates to establish which (if any) strains of *B. cepacia* are prevalent in patients with CF at various treatment centers and to identify strains that might be transmitted from patient to patient. Because of the large numbers of isolates involved, a PCR-based assay was utilized to enable high sample throughput (10, 18). We developed a RAPD typing method, which, unlike that reported previously (2), utilized several different arbitrary primers screened and selected for their ability to produce stable and discriminatory polymorphisms. The same RAPD method has been successfully applied to differentiate a large collection of *P. aeruginosa* isolates from CF patients (18). The RAPD typing results of 627

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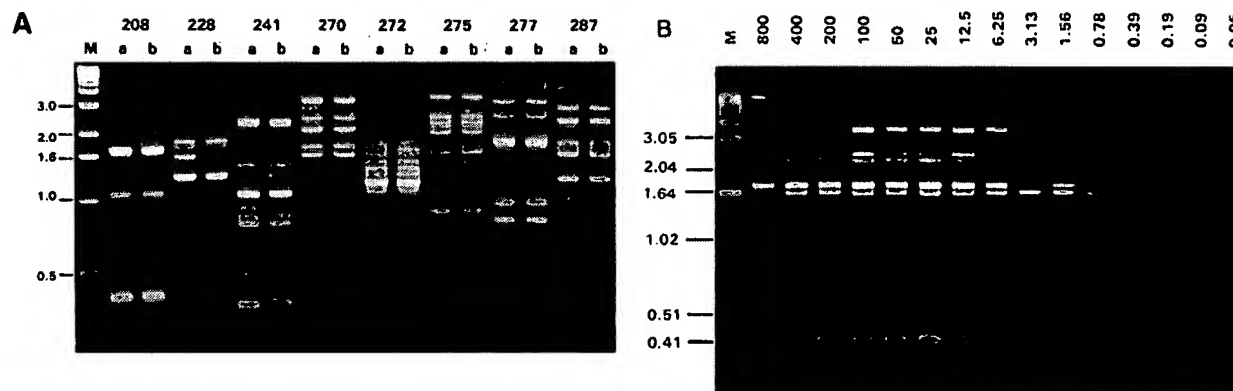


FIG. 1. (A) The polymorphisms amplified by eight RAPD primers from *B. cepacia* ATCC 25416 DNA prepared on two separate occasions (a and b) from the same frozen culture stock. Primer numbers are indicated above each lane. (B) The effect of template DNA concentration on the RAPD fingerprint profile. The amount of DNA (nanograms) added to each reaction mixture is shown above each lane; DNA extracted from *B. cepacia* FC0001 (RAPD type 4) was used and amplified with primer 208 under the conditions described in Materials and Methods. Molecular size markers were run in lane M, and their size (in kilobases) is indicated.

B. cepacia isolates from patients at several CF treatment centers and from various other clinical and environmental sources form the basis of this report.

MATERIALS AND METHODS

Collection of *B. cepacia* isolates and microbiological methods. Isolates of *B. cepacia* were received from CF clinics and clinical and research laboratories across Canada and from the United States and the United Kingdom. A total of 627 isolates were examined and included 525 isolates recovered from 255 patients with CF, 44 isolates recovered from 43 patients without CF, and 58 isolates recovered from the environment. Canadian contributors were as follows: P. Zuberbuhler and N. Brown (University of Alberta Hospitals, Edmonton, Alberta), E. Sheperd (Janeway Child Health Center, St. John's, Newfoundland), M. Ruel and L. Cote (University of Laval Hospital Center, Sainte Foy, Quebec), L. Wilcox (McMaster University Medical Centre, Hamilton, Ontario), A. Matlow (Hospital for Sick Children, Toronto, Ontario), E. Tullis (Wellesley Hospital, Toronto, Ontario), J. Hillsdon-Smith (Laurentian Hospital, Sudbury, Ontario), D. Hughes (Izaak Walton Killam Children's Hospital, Halifax, Nova Scotia), N. Cimolai (British Columbia's Children's Hospital, Vancouver, British Columbia), A. Clarke (St. Paul's Hospital, Vancouver, British Columbia), M. Li (Victoria General Hospital, Victoria, British Columbia), and W. Johnson (Laboratory Center for Disease Control, Ottawa, Ontario). U.S. contributors were as follows: J. Burns (Children's Hospital and Medical Center, Seattle, Wash.), T. Stull (Medical College of Philadelphia, Philadelphia, Pa.), M. Roy (Genentech, Inc., San Francisco, Calif.), and P. Ferrieri (University of Minnesota, Minneapolis). A collection of isolates from a variety of different sources, including CF patients in the United Kingdom, was provided by J. Govan (University Medical School, Edinburgh, Scotland, United Kingdom). Isolates from France were provided by E. Bingen (Hôpital Robert Debré, Paris, France), and isolates from Australia were provided by P. Taylor (Prince of Wales Hospital, Randwick, New South Wales, Australia).

Culture and confirmation of identification of isolates were carried out as described previously (3). After confirmation of species, epidemiological (if known) and biochemical data were entered into a computer database. All isolates were assigned a code and, after DNA extraction, were assigned a separate arbitrary number (with the prefix "B") to allow for blinding and unbiased interpretation of results. The repository code for one isolate representative of each RAPD type is shown in Tables 1, 2, and 3.

RAPD typing of *B. cepacia*. Genomic DNA was isolated from *B. cepacia* by mechanical disruption with glass beads exactly as described for *P. aeruginosa* (18). One hundred random 10-base RAPD primers were screened for their ability to amplify polymorphisms from *B. cepacia* ATCC 25416 DNA with the PCR conditions described previously (1, 18). The eight primers able to amplify polymorphisms from *P. aeruginosa* (18) were also found to produce fingerprinting profiles from *B. cepacia* (see Fig. 1A); their sequences are as follows (5' to 3'): 208, ACGGCCGACC; 228, GCTGGGCCGA; 241, GCCCGAGCGG; 270, TGCGCGCGGG; 272, AGCGGGCCAA; 275, CCGGGCAAGC; 277, AGG AAGGTGC; and 287, CGAACGGCGG. Primary typing of all the *B. cepacia* isolates described in this study was performed with primer 270, and confirmation of the strain types established with this primer was provided with primers 208 and 272. RAPD fingerprint profiles were compared visually and with the aid of computer analysis (GelManager for Windows; Biosystematica, Prague, Czech Republic) (18). Similarity coefficients were calculated across the entire absor-

bance profile of each fingerprint by a Pearson product moment coefficient (GelManager for Windows).

PCR ribotyping. The PCR primers and conditions described by Kostman et al. (11) were used to amplify the polymorphisms present in the 16S-23S spacer regions of the rRNA operon of *B. cepacia*. DNA polymorphisms were then visualized after separation of fragments on 2% agarose gels as described previously (24). Comparison of the PCR ribotyping polymorphisms was made by eye.

RESULTS

Identification of primers for RAPD analysis. The polymorphisms amplified by the eight functional RAPD primers from DNA extracted from two subcultures of *B. cepacia* ATCC 25416 made from the same freezer vial on two separate occasions are shown in Fig. 1A. Each primer amplified a DNA fingerprint ranging from 5 to 20 bands, over a size range of 100 bp to 5 kb, which was reproducible for the two independent preparations of DNA. Two conditions were found to affect the reproducibility of the RAPD fingerprint: template DNA concentration and primer concentration. The effect of the template DNA concentration on fingerprint profile is shown in Fig. 1B. The RAPD polymorphisms remained stable with 10 to 100 ng of template DNA; if less than 10 ng of *B. cepacia* DNA was present in the PCR, no or partial fingerprints were obtained, and with greater than 100 ng of DNA, there was loss of banding (Fig. 1B). RAPD fingerprints were also stable when between 10 and 160 pmol of primer was added to reaction mixtures containing 40 ng of template DNA (data not shown). Reaction mixtures containing 40 ng of template DNA and 40 pmol of RAPD primer were found to be optimal for amplification of reproducible fingerprints from *B. cepacia* and were applied throughout the study. Failure to generate a reproducible fingerprint from a sample of DNA was rare; such problems were corrected by preparation of a fresh DNA stock, and reproducible fingerprints were generated for all 627 isolates examined in the study. Although all eight primers were found to discriminate among unrelated *B. cepacia* strains (data not shown), primer 270 (the first primer to be evaluated) was used to type all the isolates examined in this study (see below). Primers 208 and 272 were used to confirm RAPD types found with primer 270; further evaluation of the remaining five primers was not carried out.

Identification of genetically related *B. cepacia* strains. All *B. cepacia* isolates were typed without prior knowledge of their source by RAPD with primer 270. Nine groups of related RAPD fingerprints and five unique fingerprints were found in

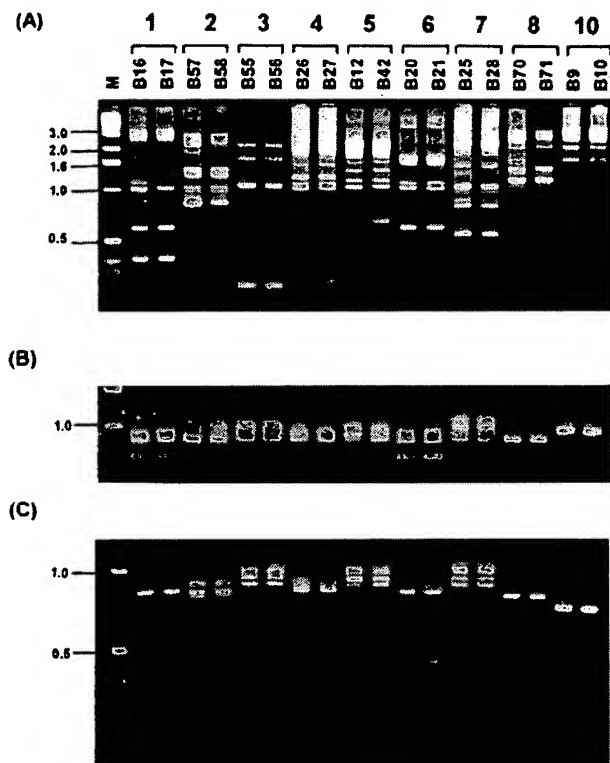


FIG. 2. RAPD fingerprint and PCR ribotype of typed *B. cepacia* isolates. (A) The RAPD fingerprints, amplified with primer 270, of nine types of *B. cepacia*. (B) The corresponding PCR-ribotype amplification patterns. (C) The *Hae*III digestion products of the PCR-ribotype polymorphisms. Isolate numbers are indicated above each lane, and the designated RAPD group number is above each pair of isolates in panel A. Molecular size markers were run in lane M, and their size in kilobases is indicated.

the first 60 isolates examined. The polymorphisms generated with primer 270 of these nine RAPD types are shown in Fig. 2A. The fingerprints generated by primer 270 enabled good primary discrimination of *B. cepacia* strain types. Within RAPD types, the amplified polymorphisms were conserved and the similarity coefficients of the banding profile were above 0.7 as determined by computer-assisted analysis. Between RAPD types, differences in the number of amplified markers, their molecular size, and intensity were considerable and enabled good interstrain discrimination.

The RAPD typing criteria applied to the first 60 isolates also enabled epidemiologically related strains to be grouped, including sequential isolates from individual CF patients and isolates of published epidemiologic and ribotype similarity (see below and Table 1). For example, RAPD types 3, 5, 6, and 7 were representative of sequential isolates each recovered from individual CF patients attending the pediatric clinic in Vancouver (type 6 was later recovered from additional CF patients [see below]). Types 8 and 10 were each representative of sequential isolates recovered from individual CF patients in Seattle, Wash. (12), and Philadelphia, Pa. (14); the published ribotype of each strain type was also distinct (12, 14). Type 1, 2, and 4 strains each infected multiple CF patients but were center related in epidemiology (see below). Isolates belonging to each epidemiologically related cluster possessed RAPD fingerprints with similarity coefficients above 0.7 and differed by no more than three bands; these criteria formed the experi-

mental basis upon which all further isolates were typed by RAPD.

To validate the strain types assigned by RAPD against another PCR typing method, the first 60 isolates were also evaluated by PCR-mediated ribotyping (11). The polymorphisms generated before and after restriction digestion with the endonuclease *Hae*III are shown in Fig. 2B and C, respectively. Within each *B. cepacia* strain type designated by RAPD, isolates also possessed a conserved PCR-ribotype fingerprint (Fig. 2B); however, PCR ribotyping did not easily distinguish among some of the RAPD-assigned types (e.g., groups 1, 4, and 6). The restriction fragment length polymorphisms obtained after digestion of the ribotype products with the enzyme *Hae*III are shown in Fig. 2C. The endonuclease-digested PCR-ribotype profiles of members of an RAPD group were also identical. The *Hae*III restriction fragment length polymorphism of the amplified PCR-ribotype polymorphisms enabled 8 of the 10 groups to be differentiated, but the polymorphisms generated for groups 4 and 6 remained similar in profile and indistinguishable. All further *B. cepacia* isolates described in this study were typed by RAPD fingerprinting.

RAPD analysis of *B. cepacia* isolates recovered from patients with CF. The results of the RAPD analysis of *B. cepacia* isolates recovered from CF patients are summarized in Table 1. A total of 525 CF isolates were analyzed, and 20 fingerprint types in which two or more isolates shared the same pattern were identified; 58 isolates possessed fingerprint profiles which were unique. Ten isolate types, 1, 2, 4, 6, 13, 15, 17, 23, 35, and 40, were recovered from two or more CF patients (Table 1). Type 15 *B. cepacia* was recovered from two pediatric patients in Vancouver (Table 1); however, the strain was subsequently not cultured from one of the patients, who became stably colonized with type 6 *B. cepacia*. Type 23 *B. cepacia* isolates were recovered from two CF patients in Oklahoma, and the type 35 isolates were recovered from two CF patients in Edinburgh; no other strains of each type were present in our collection to support their transmissibility.

Of the remaining *B. cepacia* types infecting multiple patients, each was recovered from three or more patients. Type 1 *B. cepacia* was the predominant strain type in the Vancouver pediatric CF clinic and also infected CF patients in the United States and France. Type 2 isolates were recovered from multiple CF patients in the United Kingdom and across Canada; this RAPD type included strains of published ribotype and PFGE fingerprint which belong to the epidemic *B. cepacia* lineage (7, 15, 29). Type 4 *B. cepacia* was the predominant strain among adult CF patients in Vancouver and also infected patients in Quebec and Nova Scotia. *B. cepacia* type 6 was recovered from five CF patients in Vancouver. Type 17 organisms were recovered from a CF patient in Ontario and included strains representative of the predominant *B. cepacia* ribotype infecting multiple patients at a CF treatment center in Cleveland (15). Finally, type 40 *B. cepacia* was an epidemic strain type which was recovered from 17 CF patients in an Australian treatment center.

RAPD group 2 was the most common CF strain type in our collection (267 of 525 CF isolates tested). RAPD analysis demonstrated that this strain type was widespread in Canadian CF clinics, infecting more than 100 CF patients residing in Nova Scotia, Newfoundland, Ontario, Alberta, and British Columbia (Table 1). The polymorphisms amplified by primer 270 from 38 members of this typing group are shown in Fig. 3. Minor variations in fingerprint patterns within RAPD type are illustrated in Fig. 3. The similarity coefficients of the polymorphisms were greater than 0.8, designating them as a single RAPD type. However, the isolates from Newfoundland were

TABLE 1. Summary of RAPD analysis of *B. cepacia* isolates recovered from patients with CF

RAPD type	No. of isolates	No. of patients and geographic location ^a	Repository no. (reference) ^b
01 ^c	48	9; pediatric, BC 2; adult, BC 2; Paris, FR 2; USA	C1764 C6062 CEP498 CEP156
02 ^d	267	12; UK clinics (various) 1; pediatric, BC 7; adult, BC 5; Edmonton, AL 2; IWK, NS 13; St. John's, NF 2; Sudbury, ON 9; McMaster, ON 7; CH. of E, ON 5; HSC, ON 55; Wellesley, ON	CEP125 (7) C4629 C5424 CEP274 CEP457 CEP011 CEP556 CEP051 CEP555 CEP431 CEP331
03	9	1; pediatric, BC	CEP023 (15)
04 ^c	52	4; pediatric, BC 11; adult, BC 4; IWK, NS 1; Laval, QUE 1; Oklahoma	C5393 C4813 C3938 CEP456 CEP001 CEP095
05	4	1; pediatric, BC	C514
06	23	4; pediatric, BC 1; adult, BC	C2303 C6508
07	2	1; pediatric, BC	C3430
08	14	1; Seattle, Wash.	CEP041 (12)
10	4	1; Philadelphia, Pa.	CEP021 (14)
12	2	1; pediatric, BC	C5274
13	5	3; Manchester, UK	CEP113
14 ^d	1	1; Manchester, UK	CEP222
15	3	2; pediatric, BC	C4297
17	5	1; Woodstock, ON 1; Cleveland, Ohio	CEP053 CEP024 (15)
19	2	1; pediatric, BC	C5568
23	2	2; Oklahoma	CEP087
24 ^d	3	1; Oklahoma	CEP094
33	2	1; Glasgow, UK	CEP123
35	2	2; Edinburgh, UK	CEP139
40	17	17; New South Wales, AUS	CEP529
Unique	58	57; various	
Total	525	255	

^a Abbreviations: pediatric, pediatric clinic; BC, British Columbia; adult, adult clinic; FR, France; USA, United States; UK, United Kingdom; AL, Alberta; IWK, Izaak Walton Killam Children's Hospital; NS, Nova Scotia; NF, Newfoundland; ON, Ontario; CH of E, Ottawa; HSC, Hospital for Sick Children; QUE, Quebec; AUS, Australia.

^b Repository code of a representative isolate for the RAPD type.

^c Also a clinical isolate type (see Table 2).

^d Also an environmental isolate type (see Table 3).

consistently different from the rest, having one extra band of approximately 0.7 kb (Fig. 3). Isolate B491 from the Toronto center was the only other member of *B. cepacia* type 2 to have this band. There was no difference in the PCR-ribotype polymorphisms of these isolates (Fig. 2B and C).

Epidemiology of *B. cepacia* colonization in Vancouver. A total of 58 CF patients attending clinics in Vancouver were colonized with *B. cepacia*. The pediatric and adult treatment centers were on the same hospital site separated by about 200 m until September 1993, at which time the adult CF clinic was moved to another hospital. Although the clinics were in proximity, the patients were cared for by different staff, there was little interaction between the clinics, and the patients were hospitalized in separate facilities. The prevalent *B. cepacia*

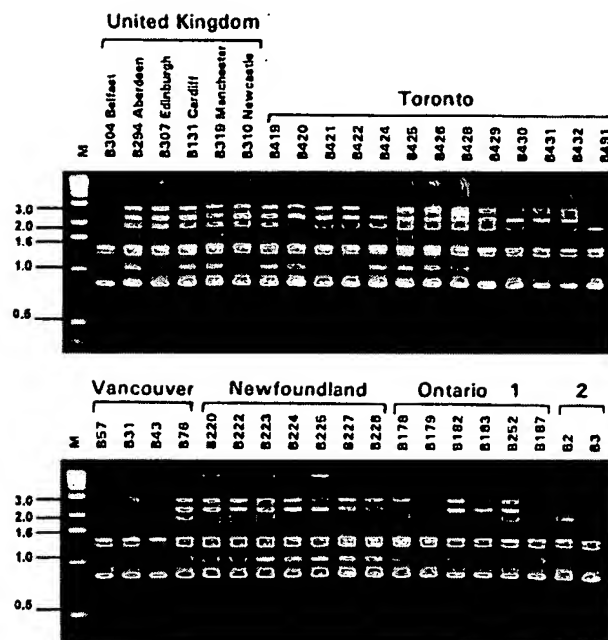


FIG. 3. The polymorphisms amplified by primer 270 from members of *B. cepacia* RAPD type 2. Isolates recovered from individual patients are shown; strain numbers and their geographic source are indicated above each lane. Isolates from Toronto were recovered from patients attending the Wellesley Hospital; Ontario 1 isolates were from the McMaster University Medical Center, and Ontario 2 isolates were from the Laurentian Hospital. Molecular size markers were run in lane M, and their size in kilobases is indicated.

strain types at each CF clinic were different (Fig. 4). *B. cepacia* type 1 predominated among patients attending the pediatric clinic (9 of 30 patients); two adult patients were colonized with this strain type. *B. cepacia* type 4 was the predominant strain among patients attending the adult CF clinic (11 of 18 patients). Two CF patients who were colonized as children with types 5 and 7 subsequently lost these strains and became colonized with type 4 after attending the adult clinic. The epidemiology of RAPD type 2, the epidemic *B. cepacia* strain (7, 29), in Vancouver was investigated. Although this type was preva-

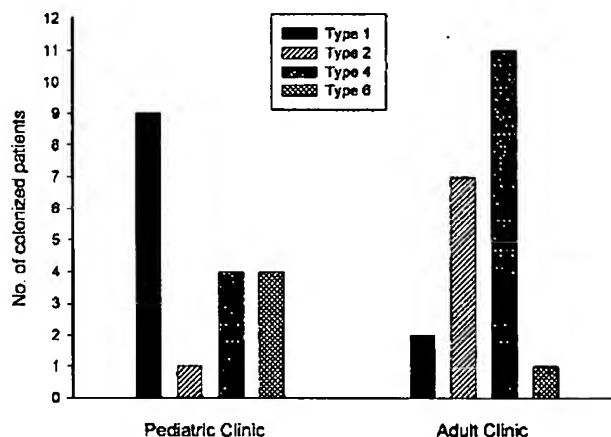


FIG. 4. *B. cepacia* RAPD types recovered from CF patients in Vancouver. The number of colonized patients at each treatment center is shown on the y axis, and the RAPD type is indicated in the key.

TABLE 2. RAPD analysis of *B. cepacia* isolates from patients without CF^a

RAPD type	No. of isolates	No. of patients	Source of infection	Geographic location	Repository no. ^b
01 ^c	2	2	ICU outbreak	UK	CEP237
04 ^c	5	5	Non-CF ^d	CDC	CEP150
	3	3	Non-CF	UK	CEP211
	3	3	Blood/ICU	Children's Hospital, BC	CEP048
09	2	1	CGD	Sacramento, Calif.	CEP108
29	3	3	Non-CF	UK	CEP231
32	4	4	Non-CF	CDC	CEP191
36	2	2	Non-CF	CDC	CEP192
38	3	3	Non-CF	UK	CEP209
39 ^e	1	1	Non-CF	USA	CEP232
Unique	16	16	Non-CF CGD	Various	
Total	44	43			

^a Abbreviations: ICU, intensive care unit; UK, United Kingdom; CDC, Centers for Disease Control and Prevention; BC, British Columbia; CGD, chronic granulomatous disease. USA, United States.

^b Strain number of representative isolate for RAPD type.

^c Also a CF isolate type (see Table 2).

^d Non-CF, culture site not known.

^e Also an environmental isolate type (see Table 3).

lent at other centers (Table 1), the number of patients colonized with this strain type attending the Vancouver treatment centers was low (8 of 58), and strains of other RAPD genotypes predominated (Fig. 4).

RAPD analysis of *B. cepacia* isolates from other clinical sources or from the environment. The fingerprinting results of clinical and environmental *B. cepacia* isolates are summarized in Tables 2 and 3, respectively. Of 44 clinical isolates, 28 fell into one of eight RAPD types and 16 were unique. *B. cepacia* types 1 and 4, major CF RAPD types, were also recovered from patients without CF (Table 2). Two type 1 strains were isolated from patients in an intensive care unit in the United Kingdom. Type 4 was also found in collections of non-CF isolates from the Centers for Disease Control and Prevention, Atlanta, Ga., and from the United Kingdom. Three isolates from separate patients without CF attending British Columbia's Children's Hospital were RAPD type 4 (Table 2); *B. cepacia* of this genotype was present among CF patients attending the pediatric clinic in the hospital and was also the predominant strain type in the adult CF clinic (Table 1 and Fig. 4). One clinical isolate was type 39, the same RAPD group as *B. cepacia* ATCC 25416 isolated from rotten onions (Table 3).

Of the 58 environmental isolates analyzed by RAPD, each of 37 fell into 1 of 15 RAPD strain types and 21 possessed unique fingerprints (Table 3). Several of the environmental isolates provided by different investigators were the same RAPD type and were obtained from the American Type Culture Collection. For example, RAPD types 30 and 39 were of replicate isolates of two American Type Culture Collection strains, 17616 and 25416, respectively, deposited in our collection by separate investigators, and matched the fingerprint of the respective type strains obtained from the American Type Culture Collection for our repository. Since typing was performed without knowledge of isolate source, matching of these identical type strains confirmed the specificity of the RAPD typing system. *B. cepacia* isolates recovered from hospital environments in Cardiff and Manchester, RAPD types 2 and 14, respectively (Table 3), were also isolated from CF patients attending these centers (Table 1). One other hospital environmental isolate

from Israel, type 24, matched the fingerprint of three *B. cepacia* isolates recovered from a CF patient in Oklahoma.

DISCUSSION

The RAPD technique we have developed enabled large numbers of *B. cepacia* isolates from our strain repository to be compared at the genetic level. RAPD was able to distinguish *B. cepacia* isolates more effectively than PCR ribotyping, consistently type serial isolates from individual CF patients, group isolates that were related epidemiologically, and appropriately distinguish isolates with known ribotype and PFGE genomic fingerprints without prior knowledge of epidemiology. Our study also provides further evidence that CF treatment centers may harbor one or more predominant strains of *B. cepacia* and that these CF isolates are, in general, genetically distinct from other clinical isolates and environmental strains.

RAPD fingerprinting was able to produce a discriminatory and reproducible genetic fingerprint from all *B. cepacia* isolates tested. We used this method to examine *P. aeruginosa* isolates recovered from CF patients and found it to be as sensitive as PFGE for typing this species once discriminatory primers were identified (18). For *P. aeruginosa* (18) and *B. cepacia*, the primer-to-template ratio was optimized; however, the PCR cycle conditions described by Akopyanz et al. (1) were unaltered. These data suggest that the original parameters described by Akopyanz et al. (1) for typing *Helicobacter pylori* are a versatile RAPD thermal cycle that may be applied to many bacterial species. Indeed, reproducible and discriminatory polymorphisms were amplified from the following bacterial species that were tested as part of our collection because they had been originally misidentified as *B. cepacia* (3): *Alcaligenes faecalis*, *Alcaligenes xylosoxidans*, *Burkholderia gladioli*, *Comamonas acidovorans*, *Enterobacter agglomerans*, and *Stenotrophomonas (Xanthomonas) maltophilia* (17a). Successful typing of *B. cepacia*, *P. aeruginosa* (10, 18), and *H. pylori* (1) with the same basic RAPD technique illustrates that such techniques are transferable from one laboratory to another and that reports of unreliable RAPD typing schemes (9) are misleading.

TABLE 3. RAPD analysis of *B. cepacia* isolates from environmental sources^a

RAPD type	No. of isolates	Source	Geographic location	Repository no. (reference) ^b
02 ^c	1	Hospital	Cardiff, UK	CEP137
11	3	Soil	Edinburgh, UK	CEP159
14 ^c	1	Hospital	Manchester, UK	CEP181
18	3	Soil	Edinburgh, UK	CEP240
21	3	Onion	USA	CEP072
22	3	Onion	USA	CEP073
24 ^c	1	Hospital	Israel	CEP195
25	3	Onion	USA	CEP076
26	2	Hospital	Cardiff, UK	CEP181
28	3	Soil	UK	CEP155
30	2	Soil	ATCC 17616	CEP144
31	2	Soil	ATCC 35130	CEP084
34	2	Plant/pond	Edinburgh, UK	CEP193 (4)
37	2	Hospital	Manchester, UK	CEP171
39 ^d	6	Onion	ATCC 25416	CEP031
Unique	21	Various	Various	
Total	58			

^a Abbreviations: UK, United Kingdom; USA, United States.

^b Strain number of representative isolate.

^c Also a CF RAPD type (see Table 1).

^d Also a clinical RAPD type (see Table 2).

RAPD analysis correctly clustered isolates of known ribotype and PFGE profile (7, 15), although direct comparison with these methods was not performed. Comparison of RAPD fingerprints with PCR-ribotyping profiles demonstrated, as others have described (17), that PCR ribotyping is not as discriminatory as RAPD for epidemiological analysis of *B. cepacia*. Kostman et al. (11) suggested that restriction digestion of the amplified products of PCR ribotyping may improve the discriminatory power of PCR ribotyping. Digestion of PCR-ribotyping products with the enzyme *Hae*III did not significantly improve the discrimination of the *B. cepacia* isolates examined in this study. RAPD has been shown to be as discriminatory as PFGE (2, 17) and enterobacterial repetitive intergenic consensus sequence PCR (17) for distinguishing *B. cepacia*; however, day-to-day variation in the fingerprints of the RAPD method evaluated (2) was found (17). Such variation was not apparent with our method, and the good reproducibility was also found with RAPD analysis of *P. aeruginosa* (18). The large amount of template DNA required for RAPD (a minimum of 10 ng [Fig. 1B]) may also be advantageous for a PCR-based assay, since contaminating DNA would not produce conflicting polymorphisms unless it represented more than 25% of the sample (40 ng was used in each PCR). In contrast, because of the specific nature of ribotyping primers (11), PCR ribotyping was able to amplify polymorphisms from as little as 10 pg of DNA (data not shown), indicating that trace amounts of contaminating DNA may interfere with the banding patterns produced by this PCR method.

Our study provides further evidence that CF centers may harbor one or more predominant *B. cepacia* strains. Steinbach et al. (27) found no evidence of strain transmission among 17 CF patients at a single treatment center and stated that previous reports (7, 15) had incorrectly suggested that CF centers generally harbor one or more transmissible *B. cepacia* strains. This statement was contested by other investigators (6, 16, 20). The broad study of CF centers presented in this study, in which several patients at each center were found to harbor the same strain type, is in agreement with previous reports (7, 15, 25) indicating the presence of center-specific predominant *B. cepacia* strains. RAPD type 2 was the most prevalent CF strain type found (Table 2). The transatlantic spread of this epidemic clone from the United Kingdom where it was originally described (7), to centers in Ontario, Canada, has been documented by others (9, 29), and a possible route of transmission via patient contact at summer camps has been reported (21). We have also shown that this type is widespread among CF patients attending treatment centers outside Ontario, suggesting that this strain type, as others have stated (29), is a hypertransmissible *B. cepacia* lineage. However, our study of *B. cepacia* isolates from Vancouver treatment centers illustrated that, despite the presence of patients colonized with the epidemic strain (RAPD type 2), other strain types clustered among the patients studied (Fig. 4). These data suggest that the inability of Steinbach et al. (27) to detect evidence of patient-to-patient spread may have been due to the nature of their patient population, which was largely referred from other centers.

The apparent spread of type 6 *B. cepacia* from one CF patient to other CF patients attending the Vancouver pediatric clinic (Table 1; Fig. 4) further demonstrates the potential risk of patient-to-patient transmission of this organism. One patient alone was chronically colonized with this type 6 strain for 6 years, suggesting low transmissibility by the criteria stated by Steinbach et al. (27). However, in year 7, type 6 was recovered from three other patients, suggesting that this strain type might have spread from one patient to another. Prolonged social contact is an identified risk factor for transmission of *B. cepacia*

(7) and may have accounted for the spread of type 6 *B. cepacia* in the pediatric clinic. Furthermore, in Vancouver, each prevalent *B. cepacia* strain type was center specific (Fig. 4), despite the proximity of the pediatric and adult clinics. This suggests that transmission of *B. cepacia* may have occurred as a result of interaction among patients attending each clinic, although a common source at each center cannot be ruled out.

After blinded typing of the isolates described in this study, the majority of strains were grouped as clonal by RAPD. Five hundred thirty-two isolates were found to belong to 37 distinct fingerprint types, and 95 isolates were unique in their RAPD profile (Tables 1, 2, and 3). CF-associated strain types were generally different from those obtained from other clinical sources and from the environment, and only 6 of the 132 strain types found (types 1, 2, 4, 14, 24, and 39) were recovered from more than one of these sources. The difference between *B. cepacia* strains recovered from CF patients and those recovered from the environment is in agreement with previous reports (4, 9). We have preliminary data which suggest that *B. cepacia* strains that are transmissible and infect multiple CF patients (types 1, 2, 4, 6, 13, 17, and 40) harbor a region of the genome, identified by RAPD with primer 272, which is generally absent from isolates colonizing single CF patients and those recovered from other sources (reference 19 and unpublished data).

In conclusion, the RAPD method reported herein is a robust fingerprinting technique which is able to amplify discriminatory polymorphisms from the genome of *B. cepacia*. The PCR-based technique was suitably versatile to enable a large collection of *B. cepacia* isolates to be screened without prior knowledge of epidemiology. The method has enabled us to establish the prevalence of various *B. cepacia* strain types colonizing CF patients treated in Vancouver and monitor the spread of problematic transmissible strain types at other treatment centers. We have also identified two further epidemic *B. cepacia* strains (types 1 and 4) which infect multiple patients in North America and Europe. These data suggest that other *B. cepacia* lineages apart from the epidemic strain type (29) may infect the multiple patients within the global CF community. This method should permit important epidemiological questions to be answered regarding the risk of patient-to-patient spread of *B. cepacia* in different environments.

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Identification and Characterization of a Novel DNA Marker Associated with Epidemic *Burkholderia cepacia* Strains Recovered from Patients with Cystic Fibrosis

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Burkholderia cepacia is a problematic pathogen that may spread among patients with cystic fibrosis (CF). One highly infectious CF strain that causes epidemics in both the United Kingdom and eastern Canada has been shown to possess both the cable pilin subunit gene (*cblA*) and a unique combination of insertion sequences. However, no genetic markers linking this strain type with other types epidemic at various centers have been identified. Using a randomly amplified polymorphic DNA (RAPD) typing scheme, we identified an apparently conserved 1.4-kb fragment in the DNA fingerprint of epidemic *B. cepacia* strains. Conservation of the DNA marker among epidemic strains was demonstrated by Southern hybridization, and its prevalence was assessed in a collection of chromosomal DNAs extracted from 627 isolates representative of 132 RAPD-defined *B. cepacia* strain types. The marker was specifically associated with seven epidemic CF strains, was absent among nonepidemic strains infecting individual patients with CF, and rare among strains recovered from the natural environment. Only one of the seven epidemic CF strain types possessed DNA homologous to *cblA*. The RAPD marker was designated the “*B. cepacia* epidemic strain marker” (BCESM). Sequence analysis of chromosomal DNA corresponding to the 1.4-kb RAPD marker revealed the presence of a putative open reading frame (ORF) with significant homology to several negative transcriptional regulators; the ORF was designated the “epidemic strain marker regulator,” or *esmR*. The BCESM DNA is the first genetic marker that has been identified to be specifically associated with and conserved among several epidemic *B. cepacia* strains which infect multiple patients with CF.

Despite its evolving role in pulmonary infection in patients with cystic fibrosis (CF), very little is known about the pathogenesis of *Burkholderia cepacia*. Colonization of CF patients with *B. cepacia* has serious clinical implications since the organism is highly virulent in certain patients (30) and is resistant to multiple antibiotics (4) and because patient-to-patient spread of the bacterium, first documented in 1990 (17), may occur. Evidence of the spread of *B. cepacia* strains among patients with CF and clustering of strain types at treatment centers has now been reported by a number of investigators (11, 18, 20); however, the factors which facilitate patient-to-patient transmission of *B. cepacia* remain poorly understood. Transmission of *B. cepacia* among CF patients may be dependent on a number of risk factors including strain type (29, 31), patient behavior and population (11), use of contaminated therapeutic devices (15), and CF treatment center practices (14).

Few *B. cepacia* virulence factors have been characterized phenotypically or studied at the genetic level, and little is known about their role in pathogenesis during CF infection. *B. cepacia* may bind to and colonize respiratory epithelia in CF patients by mechanisms similar to those described for *Pseudomonas aeruginosa* (6). Both organisms are generally motile and piliated and may adhere to the same disaccharide moiety present in many asialoglycolipids (21). *B. cepacia* also secretes a number of extracellular virulence factors including sid-

erophores, proteinases, hemolysins, and lipase (21). Evasion of the immune system may also be enhanced by the intrinsic resistance of *B. cepacia* to nonoxidative killing (27). *B. cepacia* recovered from CF patients whose clinical condition has undergone rapid deterioration binds to respiratory mucins with high affinity in vitro (23), and adherence to buccal epithelial cells may occur via both pilus-mediated and non-pilus-mediated adhesive mechanisms (24). To date, the only genetically characterized virulence factor associated with an epidemic *B. cepacia* strain type from CF patients is the cable pilus (10, 25, 29). Tyler et al. (31) recently described a novel insertion sequence (IS), IS1356, which, in association with the element IS402, may also serve as a genetic marker for the epidemic *B. cepacia* strain type with a cable pilus; the pathogenic significance of these ISs remains to be determined (31). These observations suggest that spread of the organism may also be linked to strain type; however, no genetic markers linking the *cblA*⁺ strain type and CF patient-derived strains epidemic at other treatment centers (18, 20) have been identified.

Using a PCR-based randomly amplified polymorphic DNA (RAPD) typing scheme, we typed 627 *B. cepacia* isolates recovered from CF patients and a variety of other sources (20). During the latter study, a DNA band with a conserved size was observed in the RAPD fingerprints of *B. cepacia* strain types that were epidemic (infecting multiple CF patients at certain treatment centers) and that were presumed to be transmissible. The amplified band was absent from the RAPD fingerprints obtained from nonepidemic *B. cepacia* strain types which infected individual CF patients and rarely occurred in fingerprints for isolates recovered from the environment. The marker was designated the “*B. cepacia* epidemic strain

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marker" (BCESM) because of its association with *B. cepacia* strain types infecting multiple CF patients. In this report, we describe the identification of this novel *B. cepacia* DNA marker by RAPD analysis, the prevalence of homologous DNA among a collection of 627 *B. cepacia* isolates, and sequence analysis of the BCESM DNA. The prevalence of the cable pilin subunit gene (*cblA*) (25) among the same collection of isolates is also presented. Despite the widespread use of RAPD fingerprinting for typing various microorganisms, very few of the arbitrarily amplified DNA markers have been studied. This report provides the first characterization of a RAPD marker specifically amplified from epidemic strains of *B. cepacia*.

MATERIALS AND METHODS

Bacterial isolation, strains, and culture. *B. cepacia* isolates recovered from patients with CF, patients without CF, and the environment were received from the contributors acknowledged previously (20). Microbiological culture, identification, and storage of isolates were carried out as described previously (20). *B. cepacia* C5424 (DNA isolation number B57), from which the BCESM was cloned (see below), was isolated from a CF patient in Vancouver, British Columbia, Canada; the RAPD type of the strain was type 2, and it was a member of the *cblA*⁺ (see below) major epidemic CF lineage (20, 25). *Escherichia coli* DH5aF⁺ was used to subclone *B. cepacia* DNA.

Preparation of bacterial DNA and RAPD analysis. For RAPD reactions and dot blot hybridization, genomic DNAs were extracted from the *B. cepacia* strains after mechanical disruption exactly as described previously (19, 20). RAPD fingerprinting with primer 272 (5'-AGCGGGCCAA-3') was performed as described previously (20). For restriction fragment length polymorphism (RFLP) analysis and subcloning, genomic DNAs were purified from the *B. cepacia* isolates as follows. Overnight bacterial growths from 2-ml Luria-Bertani broth cultures (grown with end-over-end rotation at 37°C in 13-ml screw-cap plastic tubes) were harvested by centrifugation. Bacterial pellets were resuspended in 200 µl of GET (50 mM glucose, 70 mM EDTA, 50 mM Tris-HCl [pH 8]) and 2.8 ml of lysis buffer (1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 8], 50 mM EDTA [pH 8]) containing 60 µg of RNase A per ml and 30 µg of proteinase K (Boehringer Mannheim, Laval, Quebec, Canada) per ml. The resulting lysate was incubated at 37°C with end-over-end rotation for 2 to 18 h prior to the addition of 1 ml of saturated ammonium acetate. Following vigorous mixing, protein and polysaccharide contaminants in the lysate were allowed to precipitate for 1 h at room temperature and were then removed by centrifugation (17,000 × g, 10 min). DNA was then collected from the lysate by ethanol precipitation (the addition of 2.5 volumes of ethanol), washed with 70% ethanol, dried under vacuum, and dissolved in 200 µl of TE (10 mM Tris-HCl [pH 8], 1 mM EDTA). The yield of DNA was approximately 100 µg per 2 ml of overnight bacterial growth.

Purification, labelling, and hybridization with the RAPD-derived BCESM probe. The 1.4-kb BCESM band amplified from *B. cepacia* C5424 (DNA number B57) with primer 272 by RAPD analysis (see Results) was separated from other DNA in the RAPD fingerprint by agarose gel electrophoresis (26); the DNA was then purified from an excised agarose slice with a glass bead DNA binding kit (Prep-A-Gene; Bio-Rad Laboratories, Mississauga, Ontario, Canada). RAPD fingerprints and genomic DNAs were probed with a 1-kb internal fragment of the 1.4-kb RAPD marker generated by endonuclease digestion with the enzyme *Pst*I. This internal probe was purified by agarose gel electrophoresis as described above, and 25 ng was labelled with 50 µCi of [³²P]dGTP (Amersham, Oakville, Ontario, Canada) with a randomly primed DNA labelling kit (Boehringer Mannheim, Laval, Quebec, Canada).

DNA amplified by RAPD analysis and endonuclease-digested *B. cepacia* genomic DNA were separated by agarose gel electrophoresis and transferred onto Hybond N⁺ nylon membranes (Amersham, Oakville, Ontario, Canada) by using conventional capillary transfer in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (26). The DNA was fixed on the membranes by exposure to UV irradiation according to the manufacturer's instructions, and the filters were prehybridized overnight at 65°C in the following hybridization buffer: 6× SSC, 0.5% SDS, 5× Denhardt's solution, and 100 µg of denatured fragmented salmon sperm DNA per ml (26). Denatured radiolabelled probe was added to fresh hybridization buffer, and hybridization was continued overnight at the same temperature. The filters were then washed at high stringency as follows: twice for 10 min at 65°C in 200 ml of 2× SSC-0.1% SDS, followed by two more washes in 200 ml of 0.1× SSC-0.1% SDS at the same temperature. Autoradiographs of the filters were obtained by exposure to X-ray film for 24 to 48 h.

Subcloning of RAPD marker and design of the BCESM PCR probes. The 1.4-kb RAPD marker from *B. cepacia* C5424 was purified as described above and was subcloned into the PCR product cloning vector pGEM-T (Promega, Fischer Scientific, Ottawa, Ontario, Canada). Cloning of the RAPD marker was confirmed by restriction endonuclease digestion with *Pst*I, and the resulting plasmid was designated pGEM-BC57. DNA sequence analysis of the ends of the BCESM

RAPD-PCR fragment was performed by dideoxy-termination PCR sequencing (CircumVent Thermal Cycle DNA sequencing kit; New England Biolabs, Mississauga, Ontario, Canada) by using the M13 forward and reverse primers. DNA sequences were analyzed manually on acrylamide gels (26).

Specific 18-base PCR primers able to amplify the 1.4-kb BCESM band were designed from the sequence internal to that of RAPD primer 272 (see Results and Fig. 4). The sequences of the BCESM-specific primers were as follows (5' to 3'): BCESM 1, CCACGGACGTGACTAACA, and BCESM 2, CGTCCATCCGAACACGAT. PCR mixtures (25 µl) containing 100 pmol of each primer, 20 ng of *B. cepacia* DNA, 250 µM (each) deoxynucleoside triphosphate, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, and 1 U of *Taq* polymerase (Canadian Life Technologies, Burlington, Ontario, Canada) were amplified on a Perkin-Elmer Cetus thermal cycler (model TC-1), as follows: 30 cycles of 1 min at 94°C, 1 min at 63°C, and 2 min at 72°C, followed by a final extension step at 72°C for 10 min. PCR products were analyzed by electrophoresis in 1.5% agarose gels as described previously (26).

Dot blot hybridization of *B. cepacia* DNA with the PCR-derived BCESM probe. For dot blot hybridization analysis, approximately 1 µg of DNA (dissolved in TE) from each of the 627 *B. cepacia* isolates was filtered under vacuum onto positively charged nylon membranes (Boehringer Mannheim) by using a 96-well dot-blot apparatus (Bio-Rad Laboratories). The BCESM DNA was amplified from *B. cepacia* C5424 by PCR with the specific primers described above and was simultaneously labelled with digoxigenin-11-uridine-5'-triphosphate (DIG-dUTP) according to the manufacturer's instructions (2a). Dot blot filters were hybridized with the DIG-labelled BCESM DNA under the same conditions described above for the radiolabelled probe. After stringent washing, the filters were developed by chemiluminescence detection with Lumi-Phos 530 according to the manufacturer's instructions (2a). After reaction with the BCESM probe, the filters were stripped of the probe by boiling them in 0.4 M NaOH for 10 min, and the filters were washed thoroughly with 2× SSC prior to hybridization with the cable pilin gene probe (see below).

Dot blot hybridization of *B. cepacia* DNA with the cable pilin subunit gene probe. A full-length cable pilin subunit gene probe was amplified by PCR from *B. cepacia* C5424 by using the primers and conditions described by Sajjan et al. (25). The 722-bp product was labelled during PCR with DIG-dUTP as described above. Prehybridization, hybridization, and detection of the filters were carried out at high stringency exactly as described above for the BCESM probes.

Subcloning and sequence analysis of *B. cepacia* chromosomal BCESM region. Southern hybridization analysis of *B. cepacia* C5424 DNA localized the BCESM DNA to a 6.0-kb fragment that was generated by digestion of C5424 DNA with *Eco*RI and *Bam*HI. Digested chromosomal DNA in this size range was purified following agarose gel electrophoresis as described above and was subcloned into *E. coli* DH5aF⁺ by using *Eco*RI-*Bam*HI-digested pUC18 (26). The resultant recombinant clones (approximately 2,000 colonies) were divided into six pools and were grown briefly, and then plasmid DNA was extracted by alkaline lysis (26). Plasmid DNA pools were screened by PCR with the BCESM primers as described above. The BCESM DNA was amplified from two of the six pools, and one of these pools was retransformed into *E. coli*. PCR with the BCESM primers was then performed directly with individual colonies from this transformation, and two plasmid clones carrying the BCESM *Eco*RI-*Bam*HI region were identified in the first 36 colonies screened. One of these plasmids was designated pTF1.2 and was used in all subsequent characterizations of the BCESM region.

After restriction mapping of the insert of pTF1.2, a 3.3-kb *Eco*RI-*Sall* fragment encoding the BCESM region was subcloned into pBluescript II SK⁻ (Stratagene, La Jolla, Calif.), generating the plasmid pTF11. A series of unidirectional nested deletions of the insert of this plasmid were created using the Erase-a-Base System kit (Promega, Madison, Wis.). Fifteen of the resulting deletion clones were sequenced by automated PCR sequencing (Applied Biosystems 377 Automated DNA Sequencer and AmpliTaq DyeDeoxy Terminator Cycle Sequencing) with the T7 primer. Sequence data were assembled and analyzed by computer software (Lasergene for Windows; DNASTAR Inc., Madison, Wis.).

Sequence homology analysis. Computer-assisted searches of the GenBank, PIR, and SWISSPROT databases were performed by using either the BLAST or the BEAUTY program (1, 32). Analysis of the putative EsmR protein was performed with the BLOCKS program (13).

Nucleotide sequence accession number. The BCESM DNA sequence has been submitted to GenBank and assigned GenBank accession number U81966.

RESULTS

Identification of a RAPD marker of conserved size in epidemic *B. cepacia* strain types. In a separate study we reported a RAPD method that was able to discriminate *B. cepacia* isolates recovered from a variety of sources including CF infection (20). Primary typing of *B. cepacia* was performed by RAPD analysis with primer 270; however, two additional RAPD primers, primers 208 and 272, were used to confirm the assignment of a given type (20). The RAPD profiles amplified by primer 272 from strains representative of the first 14 RAPD

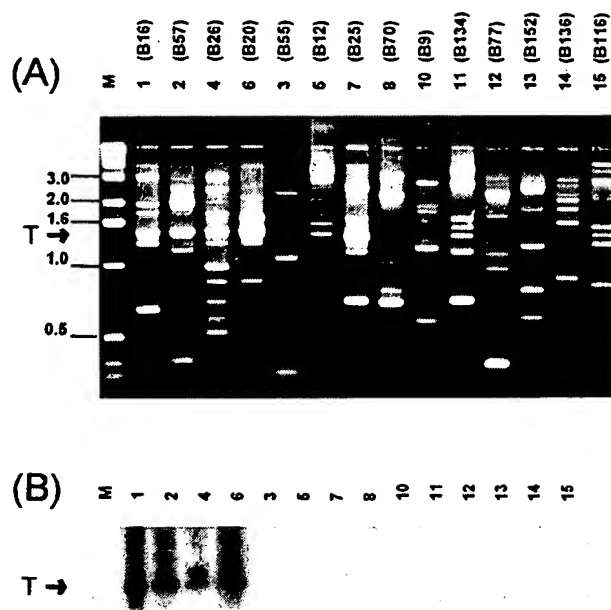


FIG. 1. RAPD fingerprints of *B. cepacia* isolates amplified with primer 272 and identification of the 1.4-kb marker. (A) DNA polymorphisms amplified from strains representative of 14 *B. cepacia* RAPD types (the RAPD type and strain number are indicated above each lane). The conserved 1.4-kb marker in RAPD types 1, 2, 4, and 6 is indicated by the letter T. Molecular size markers were run in lane M, and the sizes are indicated in kilobases. (B) Southern blot of the DNA in panel A probed with a 1.0-kb *Pst*I fragment derived from the 1.4-kb marker purified from strain C5424 (B57). The position of the hybridizing marker is indicated by the letter T.

types are shown in Fig. 1A. The fingerprint profiles of each strain type obtained with primer 272 were distinct, discriminated between strain types, and correlated with typing groups established with primer 270. However, a 1.4-kb DNA band amplified by primer 272 appeared to be conserved in the fingerprints of *B. cepacia* strain types for which there was evidence of patient-to-patient spread among CF patients in Vancouver (types 1, 2, 4, and 6; Fig. 1A) and absent from the other nine nonepidemic CF strain types and one environmental strain type (Fig. 1A).

B. cepacia type 1 was the predominant strain type colonizing 9 CF patients attending the pediatric clinic in Vancouver, type 2 was the epidemic strain type affecting more than 120 patients at treatment centers in the United Kingdom and across Canada, type 4 was the predominant strain type among patients attending the adult CF clinic in Vancouver, and type 6 *B. cepacia* infected a total of 5 pediatric CF patients in Vancouver (20). Types 3, 5, 7, 8, 10, 12, and 14 (Fig. 1A) were each isolated from individual CF patients only (20); *B. cepacia* type 15 stably colonized one CF patient and was cultured on only one occasion from another CF patient who later became stably colonized with a type 6 strain (20). Type 13 isolates were recovered from three CF patients in Manchester, United Kingdom (20); however, the 1.4-kb marker was not apparent in the fingerprint profile in Fig. 1A of the representative isolate amplified by RAPD analysis. Type 11 *B. cepacia* was isolated from soil (20). Although the 1.4-kb marker was conserved in size among the majority of isolate types which infected multiple CF patients, because of the arbitrary nature of RAPD analyses, it

may not have been homologous among the different strain types.

Conservation of the *B. cepacia* epidemic strain marker. Conservation of the BCESM DNA was demonstrated by the following experiments. The 1.4-kb DNA fragment was purified from the RAPD fingerprints of type 1, 2, 4, and 6 *B. cepacia* strains, and endonuclease cleavage of the DNA with *Hae*III and *Pst*I revealed identical RFLP profiles (data not shown). Conservation of the RFLP profile suggested that the chromosomal region amplified by primer 272 in these epidemic *B. cepacia* strain types was homologous. The absence of the 1.4-kb marker from the nonepidemic CF isolates and the environmental isolate examined (Fig. 1A) suggested that this chromosomal region was either missing or rearranged such that amplification by PCR was not possible. To further assess this chromosomal locus, both the randomly amplified DNA and the chromosomal DNA from *B. cepacia* were probed by Southern hybridization for the presence of sequences homologous to the 1.4-kb RAPD marker.

Southern hybridization analysis of the RAPD gel shown in Fig. 1A with a probe derived from the 1.4-kb RAPD marker of *B. cepacia* C5424 is shown in Fig. 1B. The 1.4-kb bands from all the *B. cepacia* isolates representative of the epidemic strain types except for type 13 (see below) hybridized to the probe (types 1, 2, 4, and 6); homologous DNA was not present in the RAPD fingerprints of the other strain types in Fig. 1A, indicating that the marker band was not amplified, even at a different size range, for these isolates. Southern hybridization of genomic DNA digested with *Pst*I also revealed the presence of homologous 1-kb DNA in epidemic strain types 1, 2, 4, and 6 (data not shown); no homologous DNA at any size range was detected in the nonepidemic strain types examined (data not shown). Examination of restriction enzyme-digested genomic DNA from *B. cepacia* C5424 by Southern hybridization localized the marker DNA to a 6-kb *Eco*RI-*Bam*HI chromosomal fragment (data not shown). Because the 1.4-kb RAPD fragment had been localized to a chromosomal locus and was truly absent from the nonepidemic CF isolates examined, the marker was designated BCESM and was cloned in *E. coli* for further characterization (see below).

Subcloning of BCESM RAPD band and design of specific PCR primers. In order to develop a specific PCR probe for the BCESM region and to subsequently facilitate cloning of the chromosomally encoded marker region, the 1.4-kb RAPD-PCR marker amplified from strain C5424 was cloned in *E. coli*, generating plasmid pBC57 (see Materials and Methods). Sequence analysis of each end of the cloned 1.4-kb PCR fragment enabled the design of specific PCR primers by using the sequences internal to that of RAPD primer 272 which formed the ends of the amplified marker (see Fig. 4). These specific 18-base PCR primers, BCESM 1 and BCESM 2 (see Materials and Methods), enabled the 1.4-kb marker to be amplified from the chromosomal DNAs of positive *B. cepacia* strains (Fig. 2).

Amplification of the BCESM by RAPD analysis from strains of types 1, 2, 4, and 6 had been demonstrated by Southern hybridization (Fig. 1), and these strain types were also positive for the specific PCR marker (Fig. 2). Type 13 isolates, which lacked the marker by RAPD analysis, but which had the characteristics of an epidemic *B. cepacia* strain, were found to amplify a single fragment by specific PCR (Fig. 2). The size of the marker amplified from type 13 isolates was slightly higher than that of the other epidemic types; however, the RFLP profile generated by digestion with *Hae*III was conserved (Fig. 2) except for the largest cleavage product, suggesting that the BCESM of type 13 strains may contain minor DNA sequence variations in this region. Type 17 isolates, the predominant CF

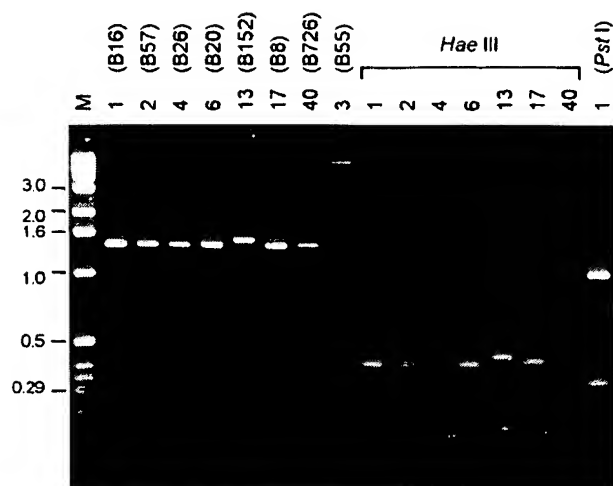


FIG. 2. Specific amplification of the BCESM DNA from transmissible *B. cepacia* strain types by PCR. The 1.4-kb BCESM DNA was amplified with the specific primers BCESM 1 and BCESM 2 and the conditions described in the Materials and Methods. One-tenth of each PCR mixture was loaded for types 1, 2, 4, 13, 17, and 40 (RAPD type and strain DNA number are indicated above each lane); half of the PCR mixture was loaded for the negative control type 3 (chromosomal template DNA forms the band at the top of this lane). The products obtained after *Hae*III digestion of the BCESM band amplified from each type and the *Pst*I digestion products of the marker from a type 1 strain (B16) are indicated on the right. Molecular size markers are shown in lane M, and the sizes are indicated in kilobases.

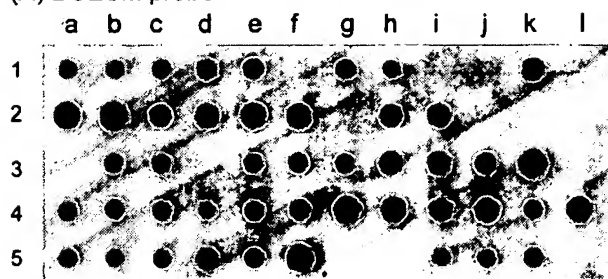
strain type at a treatment center in Cleveland, Ohio (18), and type 40, an epidemic *B. cepacia* CF strain from a center in Australia (20), were also positive for the marker (Fig. 2). No product was observed after amplification of DNA from the nonepidemic CF isolates (amplification of the DNA of a type 3 isolate is shown in Fig. 2). The BCESM DNA amplified by specific PCR shared the same *Hae*III-derived and *Pst*I-derived RFLP pattern as the RAPD marker (Fig. 2) and also hybridized to the RAPD-derived fragment (data not shown), demonstrating that both types of probe detected the same chromosomal sequence.

The specific PCR primers also facilitated the cloning of chromosomal BCESM DNA from *B. cepacia* C5424 into *E. coli* with the vector pUC18 (26) (see Materials and Methods).

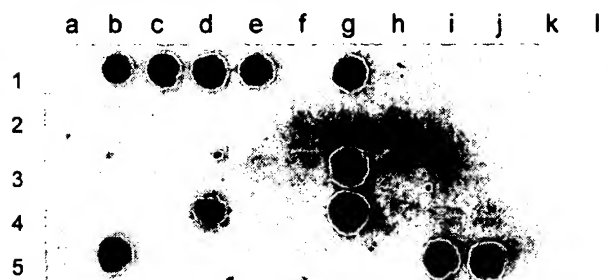
Prevalence of BCESM DNA among *B. cepacia* isolates recovered from a variety of sources. To assess the prevalence of the BCESM region among *B. cepacia* isolates, total genomic DNA from 627 strains, previously assessed by RAPD analysis (20), was examined by DNA dot blot hybridization with the specific PCR-derived BCESM probe from strain C5424 (Fig. 2, DNA sample B57); the results are summarized in Table 1. Hybridization was carried out at high stringency in order to identify sequences homologous to the BCESM probe (see Materials and Methods), and the signals obtained from the DNAs of control strains known to be positive for the marker were strong (Fig. 3, DNA samples B16, B20, B26, and B57). The BCESM PCR probe hybridized with the DNAs of all 128 CF isolates which belonged to the epidemic RAPD types 1, 4, 6, and 13. Of the three other *B. cepacia* types which infected multiple CF patients (types 2, 17, and 40), DNAs from a total of five *B. cepacia* isolates belonging to these fingerprint types did not hybridize to the marker (Table 1); two type 2, one type 17, and two type 40 isolates were negative for BCESM DNA (Table 1).

Of the remaining CF isolates examined, 101 were recovered from a total of 57 individual CF patients and were not shared

(A) BCESM probe



(B) cable pilin gene probe



(C) Strain DNA number

	a	b	c	d	e	f	g	h	i	j	k	l
1	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
2	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22	B23	B24
3	B25	B26	B27	B28	B29	B30	B31	B32	B33	B34	B35	B36
4	B37	B38	B39	B40	B41	B42	B43	B44	B45	B46	B47	B48
5	B49	B50	B51	B52	B53	B54	B55	B56	B57	B58	B59	B60

FIG. 3. Dot blot hybridization of *B. cepacia* DNA. The autoradiographs obtained after hybridization of DNA from the first 60 *B. cepacia* isolates with the BCESM PCR probe (A) and the cable pilin probe (B) are shown. The strain DNA number is indicated in panel C. Probe labelling and hybridization were as described in the Materials and Methods.

by another patient (nonepidemic CF patient-derived and unique CF patient-derived types; Table 1) (20). Three of the types classified as nonepidemic were recovered from two CF patients. Infection of the two CF patients with type 15 *B. cepacia* isolates was transient (20). Type 23 and type 35 *B. cepacia* isolates were each recovered from two CF patients at treatment centers in Oklahoma City and Edinburgh, United Kingdom, respectively; no further isolates of these types were found in the collection to support their categorization as epidemic (20). Among the isolates recovered from patients without CF, 20 of 44 isolates (45%) possessed homologous DNA (Table 1). Only 5 of the 58 isolates (8.6%) recovered from environmental sources harbored DNA homologous to the BCESM probe (Table 1).

Total genomic DNAs from the following organisms did not hybridize to the BCESM probe (*n* indicates the number of strains tested): *Agrobacterium radiobacter* (*n* = 1), *Alcaligenes faecalis* (*n* = 1), *Alcaligenes xylosoxidans* (*n* = 5), *Burkholderia gladioli* (*n* = 13), *Candida* spp. (*n* = 1), *Comamonas acidovorans* (*n* = 5), *Enterobacter agglomerans* (*n* = 1), *E. coli*

TABLE 1. Hybridization of the BCESM probe to DNA isolated from *B. cepacia* isolates recovered from a variety of sources

Source and RAPD type ^a	No. of isolates	No. of isolates with the following hybridization result:		% Prevalence of positive strains
		Positive	Negative	
Epidemic CF RAPD types (no. of patients colonized) ^b				
1 (15)	48	48	0	100
2 (>119)	267	265	2	99
4 (21)	52	52	0	100
6 (5)	23	23	0	100
13 (3)	5	5	0	100
17 ^c (>2)	5	4	1	80
40 (17)	17	15	2	88
Nonepidemic CF RAPD types ^d 3, 5, 7, 8, 10, 12, 14, 15, 19, 23, 24, 33, and 35	50	0	50	0
Unique CF isolates ^e	58	8	50	16
Clinical ^f RAPD types 1, 4, 9, 29, 32, 36, 38, and 39	28	13	15	46
Unique clinical isolates ^e	16	7	9	43
Environmental ^g RAPD types 2, 11, 14, 18, 21, 22, 24, 25, 26, 28, 30, 31, 34, 37, and 39	37	3	34	6
Unique environmental isolates ^e	21	2	19	9
All isolates				
CF isolates	525	420	105	80
Clinical isolates	44	20	24	45
Environmental isolates	58	5	53	12
Total	627	445	182	71

^a Adapted from RAPD typing data described by Mahenthiralingam et al. (20).^b RAPD type infecting more than three CF patients.^c Type 17 isolates were the dominant strain ribotype among patients attending a Cleveland CF treatment center (18); however, the 5 isolates present in our collection were representative of isolates recovered from only two of these patients.^d RAPD type infecting individual CF patients at the time of data collection and isolate culture.^e Strains with unique RAPD fingerprints unmatched in our collection at the time of data collection.^f *B. cepacia* strains isolated from patients without CF.^g *B. cepacia* strains recovered from the environment.

(*n* = 2), *Chryseobacterium* (*Flavobacterium*) *meningosepticum* (*n* = 1), *P. aeruginosa* (*n* = 15), *Mycobacterium tuberculosis* (*n* = 2), and *Stenotrophomonas* (*Xanthomonas*) *maltophilia* (*n* = 4) (data not shown).

Correlation of the BCESM marker and *B. cepacia* RAPD type. Since the majority of CF isolates in our collection belonged to the epidemic CF RAPD types 1, 2, 4, 6, 13, 17, and 40 (417 of 525 CF isolates; Table 1), the bias in total numbers

may have skewed the distribution of the BCESM probe. However, because all 627 isolates had been typed by RAPD analysis, the distribution of the BCESM DNA was also examined by strain type; these data are summarized in Table 2.

In total, 78 distinct RAPD types of *B. cepacia* were recovered from CF patients, and 15 of these possessed BCESM DNA (Table 2). Of the positive strain types, seven types were epidemic and infected multiple CF patients, as explained

TABLE 2. Prevalence of BCESM DNA and *cblA* DNA among *B. cepacia* RAPD strain types

RAPD type ^a	Total no. of strains	No. BCESM positive (RAPD type)	No. <i>cblA</i> positive (RAPD type)
CF epidemic type	7	7 (1, 2, 4, 6, 13, 17, and 40)	1 (2)
CF nonepidemic type	13	0	0
CF unique type	58	8	1
Clinical type ^{b,c}	8	3 (1, 4, and 38)	0
Clinical unique type	16	4	0
Environmental type ^d	15	2 (2 and 26)	2 (2 and 31)
Environmental unique type	21	2	3
Total ^e	132	23 [17.4%]	6 [4.5%]

^a Adapted from RAPD typing data described by Mahenthiralingam et al. (20).^b Both clinical and CF isolates of RAPD types 1 and 4 were included in this category.^c Both clinical and environmental isolates of RAPD type 39 were included in this category.^d Both CF and environmental isolates of RAPD types 2, 14, and 24 were included in this category.^e The totals were calculated after subtraction of the six strain types recovered from more than one source, which are described in footnotes b, c, and d.

above; the eight remaining positive types had a unique fingerprint, and each was recovered from an individual CF patient in disparate geographical locations (20). Therefore, in contrast to the data obtained from the total number of isolates, the majority of CF strain types lacked BCESM DNA. Sixty of the negative CF strain types (10 nonepidemic CF types and 50 unique CF types; Table 2) were each recovered from individual CF patients, and no evidence of spread of these strain types was apparent. Of the three remaining BCESM-negative CF strain types (types 15, 23, and 35), evidence of patient-to-patient spread was not substantiated by further data (20).

Among the *B. cepacia* strain types recovered from patients without CF, 7 of a total of 24 types possessed the marker (Table 2). Of the 36 strain types recovered from the environment, 4 possessed DNA homologous to the BCESM probe. Two of these BCESM-positive environmental strain types were isolated from hospital environments (types 2 and 26; Table 2) (20). The remaining two BCESM-positive types were recovered from the natural environment (20).

Prevalence of the cable pilus gene, *cblA*. The DNAs from all 627 *B. cepacia* isolates were also hybridized with the cable pilus gene probe (*cblA*) (25). Dot blot hybridization results for DNA from the first 60 isolates are presented in Fig. 3B. In total, 272 of the 627 isolates tested possessed DNA homologous to the *cblA* gene, and of these, 267 (98%) belonged to the same strain lineage, RAPD type 2, the major CF RAPD strain type (20) representative of this epidemic lineage (29, 31). None of the other epidemic CF strain types or nonepidemic CF strain types possessed DNA homologous to *cblA*. One CF isolate with a unique RAPD fingerprint, recovered from a patient in Oklahoma, hybridized with the *cblA* probe; DNA from this isolate did not hybridize with the BCESM probe (Table 2). The *cblA* gene was present in *B. cepacia* ATCC 35130, an environmental isolate, and another environmental isolate from Mexico which shared the same RAPD type (type 31) as ATCC 35130 (Table 2). Two further environmental isolates from distinct geographical sources (the United States and the United Kingdom) with unique RAPD fingerprints possessed cable pilus DNA; both of these lacked DNA homologous to the BCESM probe. None of the clinical isolate types recovered from patients without CF carried the cable pilus gene (Table 2). In total, only 6 of the 132 RAPD-defined *B. cepacia* strain types possessed DNA homologous to the cable pilin subunit gene (Table 2).

Nucleotide sequence of the BCESM DNA. A 6.0-kb chromosomal DNA fragment from *B. cepacia* C5424 (B57) encoding BCESM was cloned in *E. coli*; the nucleotide sequence from 3.4 kb of DNA encompassing the BCESM probe was determined (see Materials and Methods). The features of the 1,600 bp of DNA spanning the BCESM marker are shown in Fig. 4A, and the corresponding nucleotide sequence is shown in Fig. 4B.

Sequences identical to those of PCR primers BCESM 1 and BCESM 2 were found 1,419 bp apart, and recognition sites for the endonuclease *Pst*I, used to generate the first RAPD-derived 1.0-kb probe, were encoded 1,022 bp apart within the 1,419-bp PCR product (Fig. 4). The sequence of RAPD primer 272 (5'-AGCGGGCCAA-3'), which originally amplified the BCESM DNA, was only partially matched upstream and downstream of the sequences encoding the specific BCESM PCR primers (Fig. 4A). A total of 8 bp upstream and 9 bp downstream were matched at the 3' terminus of the primer binding site (Fig. 4B). Although this level of homology was sufficient for amplification of the marker from most of the epidemic strain types under the low-stringency RAPD-PCR conditions, the degree of mismatch may explain the inability of RAPD analysis to amplify the marker from type 13 strains,

which may contain further sequence differences in this region (Fig. 1 and 3).

Sequence homology analysis of the BCESM DNA. Sequence analysis of the marker revealed the presence of a 834-bp open reading frame (ORF) encoding a putative 277-amino-acid protein which was internal to the 1.4-kb BCESM marker (Fig. 4). Comparison of the sequence of this ORF with nucleotide and protein sequences present in the standard databases demonstrated significant homology to several negative transcriptional regulators. The putative ORF was designated the "epidemic strain marker regulator," or *esmR*, because of the homology to negative transcriptional regulators. The putative EsmR protein possessed the highest similarity (of 50 to 60% over several domains) with YhcK, a hypothetical transcriptional regulator from *E. coli*; UxuR, a regulator of glucuronate metabolism in *E. coli* (2, 3); PdhR, a negative transcriptional regulator of the pyruvate dehydrogenase complex of *E. coli* (28); and LldR, a putative regulator gene of *E. coli* involved in lactate dehydrogenase production (8). The region of highest similarity occurred in the N-terminal domains of all of the homologs, in which a helix-turn-helix domain was present. The putative EsmR protein also possessed a helix-turn-helix domain (Fig. 4B), suggesting that it is involved in a regulatory capacity in *B. cepacia*. Thus, EsmR likely belongs to the GntR family of bacterial regulatory proteins (12).

DISCUSSION

B. cepacia infection in patients with CF is problematic. Knowledge of the risk factors leading to transmission and the features of *B. cepacia* which may enable it to spread from one CF patient to another is limited. We have identified by RAPD analysis a novel region of DNA specifically associated with epidemic strain types of *B. cepacia* which infect multiple CF patients at various treatment centers. The identification of this marker by RAPD analysis illustrates the versatility of the PCR-based technique for the genomic characterization of organisms for which genetic knowledge is limited. Identification of a putative ORF within the marker DNA sequence, which shares homology with a family of negative transcriptional regulators, may provide insights into the virulence factors responsible for patient-to-patient spread, aid in the identification of problematic strain types, and assist in the diagnosis and management of *B. cepacia* infections in patients with CF.

To date, only the cable pilus and mesh pilus virulence factors have been linked with epidemically transmitted CF isolates of *B. cepacia* (10, 29). Goldstein et al. (10) categorized *B. cepacia* CF isolates as "epidemically transmitted" and "nonepidemically" or "low-level transmissibility" isolates from their epidemiological backgrounds. Strains possessing the cable and mesh pilus types were categorized with "epidemically transmitted" CF isolates (10). Such division of *B. cepacia* strains must be approached with caution since the risk factors for *B. cepacia* transmission and the pathogenesis of the organism are not fully understood. Tyler et al. (31) recently described IS markers which were also specifically associated with cable pilus-linked epidemic lineage. The BCESM DNA identified in this study is predominantly associated with strains of *B. cepacia* that infect multiple CF patients at various treatment centers and that by definition were epidemic at each center. However, since the available epidemiological data on the colonized patients was limited, assumptions on the exact mode and frequency of transmission of these epidemic *B. cepacia* strain types cannot be accurately made.

The *B. cepacia* epidemic strain types infecting multiple CF patients attending the Vancouver, Cleveland, Manchester, and

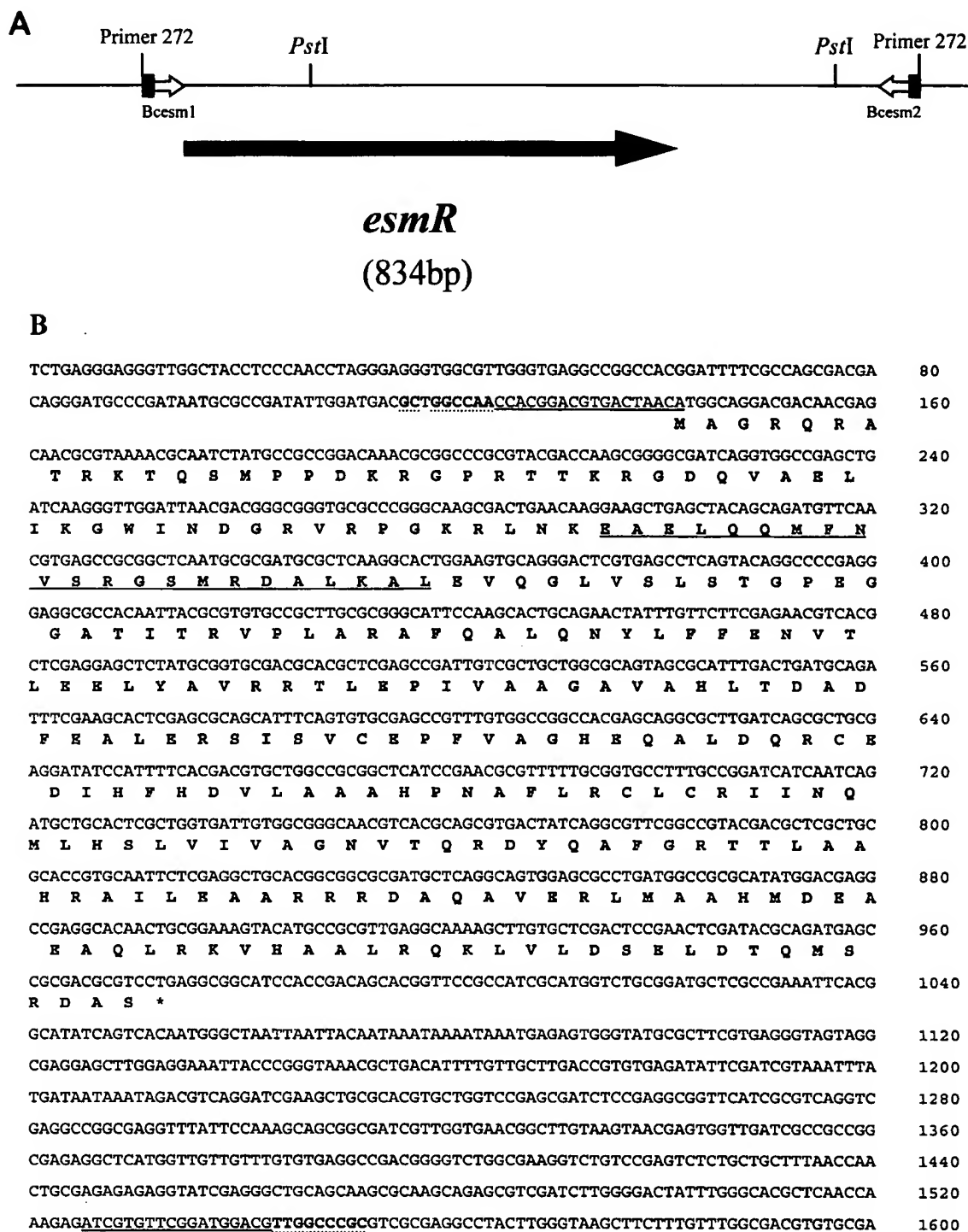


FIG. 4. BCESM chromosomal locus of *B. cepacia* C5424. (A) Features of 1,600 bp of the nucleotide sequence encompassing the marker. The RAPD primer binding sites, the BCESM primer binding sites, the *Pst*I cleavage sites, and the orientation of a putative ORF, *esmR*, are shown. (B) The corresponding 1,600-bp nucleotide sequence and amino acid translation of the putative ORF. Nucleotides matching RAPD primer 272 are indicated with a dotted line, and the sequences of the BCESM PCR primers are underlined. The amino acid translation of the putative EsmR protein is indicated by boldface type beneath the nucleotide sequence. The underlined region of EsmR corresponds to the putative helix-turn-helix domain homologous with the helix-turn-helix domains of a number of negative transcriptional regulators (12).

Australian treatment centers all lacked the cable pilus gene, but did possess DNA homologous to the BCESM (types 1, 4, 6, 13, 17, and 40; Table 1). The absence of the cable pilus marker in all the other epidemic strain types examined in this

study is in contrast to the results of Sun et al. (29), whose studies highlighted the spread of only one strain type among CF patients and promoted the fact that the *cblA* DNA is a useful marker for infectious strain types. Our data demonstrate

that all *B. cepacia* colonization among patients with CF is of major concern and that strain types other than those with the *cblA*⁺ lineage should not be considered less of a problem just because the cable pilus-carrying strain has been designated "highly infectious" (10, 30). Although the *cblA* marker is associated with the most prevalent *B. cepacia* CF strain type in the United Kingdom and North America (RAPD type 2; Tables 1 and 2) (20), it was present in only 6 of the 132 RAPD-defined strain types in the collection of 627 isolates examined (Table 2); of these 6 *cblA*⁺ types, only 1 was an epidemic CF strain type (type 2), as defined by our collection. Interestingly, *B. cepacia* RAPD type 2 was the only strain type in the entire collection possessing both the cable pilin subunit gene and the BCESM DNA. Since the combination of IS1356 and IS402 is also associated with this infectious strain type (31), these data suggest virulence factors linked with this unique combination of genetic markers may contribute to the striking prevalence of this lineage within the CF patient population.

DNA homologous to the BCESM probe was absent from 5 of 417 isolates belonging to epidemic CF types, suggesting that the region may be subject to some instability. Multiple replicons are present in the genome of *B. cepacia* (7, 22), and therefore, it is possible that the BCESM region may be encoded on a replicon which may be unstable in some transmissible CF isolates. There was no correlation with the BCESM marker and the presence of a plasmid when conventional alkaline lysis (26) was used as a means of DNA preparation (data not shown); however, this procedure is inefficient for the purification of plasmids greater than 50 kb in size (26). The genome of *B. cepacia* may also be quite plastic due to large numbers of ISs present (9), and specific ISs have recently been linked to the *cblA* epidemic strain type (31). No sequences homologous to IS elements present in databases were found on the sequence of the BCESM region; however, if IS elements flank the BCESM region that we have characterized, this may contribute to its instability in vitro in certain strain types.

The majority of CF strain types lacked the BCESM, and hence, the DNA cannot be considered a universal marker for the ability of *B. cepacia* to colonize and cause infection in patients with CF. However, the specific association of the BCESM DNA with strain types which infect multiple CF patients suggests that it can be used to identify strain types which have a high capacity to spread among CF patients and become epidemic at a given treatment center. For example, type 6 *B. cepacia*, a BCESM-positive strain, colonized only one CF patient at the treatment center in Vancouver for 6 years (20). In the seventh year of study, this strain type spread to two other pediatric CF patients and subsequently to more patients to infect a total of five CF patients attending the treatment center (20). Because of this specific association with strain transmission and not colonization per se, the BCESM DNA may mark a region of the *B. cepacia* genome which encodes virulence factors or regulators which play a role in strain transmissibility. The role of the putative EsmR protein encoded on the 1.4-kb BCESM DNA remains to be determined. Significant homology to the GntR family of bacterial regulatory proteins and possession of a DNA-binding helix-turn-helix motif (12) suggest that it may play a regulatory role in *B. cepacia*; however, the ORF's authenticity and regulatory targets remain to be determined.

Phenotypic examination of *B. cepacia* strains recovered from the natural environment has indicated that they are markedly different from epidemic strains associated with pulmonary infection in CF patients (5), and multilocus enzyme electrophoresis has also shown that environmental isolates produce electrophoretic profiles that are in general distinct from those

of CF isolates (16). The low prevalence of the BCESM DNA among *B. cepacia* strains recovered from the natural environment (2 of 36 types examined; Table 2) suggests that in general these strain types are also genetically distinct from the strains which infect multiple CF patients. Identification of the BCESM DNA region, which is the first genetic marker conserved among several epidemic types of *B. cepacia*, suggests that phenotypic factors which are associated with this DNA but which are absent from isolates present in the natural environment may be important for the spread of this organism among patients with CF.

The *B. cepacia* BCESM DNA did not hybridize to DNA from a wide variety of other microorganisms present in sputum, and the clear distinction between positive and negative *B. cepacia* strains suggests that DNA probes based on this region may be useful clinically for the identification of potentially problematic strain types. The prevalence of the *B. cepacia* BCESM marker will need to be evaluated in other CF treatment centers where transmissible isolates are thought to be present. However, on the basis of the results presented in this report, the BCESM probe is the first genetic link identified between several epidemic strain types, and it may serve as a putative marker for the presence of strains of *B. cepacia* with the potential for spread among patients with CF.

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Characterization of Hemolysin in Extracellular Products of *Pseudomonas cepacia*

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Pseudomonas cepacia is recognized as an opportunistic pathogen in immunocompromised patients. We screened 120 strains of *P. cepacia* isolated from clinical specimens for production of extracellular products. About 70% of these strains produced lipase, protease, and lecithinase, but only 4% produced hemolysin. A hemolysin produced by *P. cepacia* JN106 was characterized. The hemolysin was most active against human erythrocytes. Horse, sheep, chicken, and rabbit erythrocytes were also susceptible. The hemolysin was heat labile and was inhibited by sterols but was not activated by 2-mercaptoethanol and dithiothreitol. Four hemolysin-negative mutants obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment produced the other extracellular products. A 58-kilobase-pair plasmid found in the parent strain was also found in the mutant strains, suggesting that the hemolysin gene resides on the chromosome.

In the genus *Pseudomonas*, *Pseudomonas cepacia* has the most versatile ability to catabolize various organic compounds (24). It is highly resistant to many antibiotics and disinfectants and has been isolated with increasing frequency from clinical specimens and hospital environments (3, 6, 20, 27). The species is recognized as an opportunistic pathogen and is associated with various types of nosocomial infections (4, 7, 22, 27). In addition, *P. cepacia* now complicates cystic fibrosis (13).

There are few reports on the virulence factors of *P. cepacia*. McKevitt and Woods (19) have reported the production of virulence factors by 48 strains of *P. cepacia* isolated from patients with cystic fibrosis. A majority of strains produced protease and lipase, and about one-half of the strains produced smooth lipopolysaccharide. On the other hand, none of the strains produced elastase, cytotoxins, or ADP-phosphoribosyl transferase.

This study was initiated to determine the virulence factors of *P. cepacia*. One hundred and twenty strains isolated from clinical specimens were screened for extracellular products. In addition, a hemolysin produced by one of the strains was characterized.

MATERIALS AND METHODS

Bacterial strains. One hundred and four strains of *P. cepacia* isolated from clinical specimens in Juntendo University Hospital, Tokyo, Japan, from 1983 to 1984 were generously provided by T. Oguri and J. Igari. Among 79 strains of known origin, 15 strains were isolated from sputum, 12 strains from pus, 11 strains from urine, and 9 strains from cerebrospinal fluid; and 4 strains each were from blood, catheters, and drain tubes. Other strains were provided by S. Oie, Yamaguchi University Hospital, Ube, Yamaguchi, Japan, and E. Yabuuchi, Gifu University, Gifu, Japan. Identification of strains was confirmed by biochemical tests (9). Strains were stored in sterile glycerol solution (10% [wt/vol]) at -80°C.

Detection of extracellular products. Protease (28), elastase (23), and lecithinase (8) were determined by plate assays;

lipase production was determined by Tween 80 hydrolysis (26); and hemolysin production was determined by using plates containing 7.5% defibrinated sheep blood in heart infusion agar (Eiken Co. Ltd., Tokyo, Japan). Results were determined after incubation at 28°C for 48 h.

Preparation of crude hemolysin. Cells were grown in nutrient broth (Difco Laboratories, Detroit, Mich.) or dialysate of brain heart infusion broth (Eiken Co.) for about 48 h at 28°C with shaking. When the A_{660} of the culture reached 1.5, the culture supernatant was obtained by centrifugation (9,000 × *g*, 10 min). Crude hemolysin was prepared by ammonium sulfate fractionation and by taking the fraction between 20 and 60% saturation. This fraction was then dialyzed against 10 mM Tris hydrochloride (pH 7.4). The preparations of culture supernatant and crude hemolysin were stored at -80°C until use. Protein concentrations were determined by the method described by Lowry et al. (18).

Hemolysin assay. The hemolysin assay mixture contained 10 mM Tris hydrochloride (pH 7.4)-160 mM NaCl (hemolysin assay buffer), 2% suspensions of sheep erythrocytes that had been washed with saline, and an appropriate volume of sample containing hemolysin in a total volume of 2 ml. Control experiments for spontaneous lysis or complete lysis were carried out without hemolysin and with 0.2% sodium dodecyl sulfate, respectively. Reaction mixtures were incubated at 37°C for 10 min and chilled on ice for 2 min to stop the reaction. The unlysed erythrocytes were removed by centrifugation at 1,500 × *g* for 2 min, and the A_{530} of the supernatant was determined. The activity resulting in 50% hemolysis of 2 ml of 2% sheep erythrocyte suspensions was defined as 1 hemolytic unit (HU).

Studies on effects of various reagents on hemolysin. The culture supernatant of strain JN106 (8 HU/ml) was preincubated at 0°C for 2 h with *N*-ethylmaleimide (1 mM), *p*-chloromercuribenzoate (1 mM), 2-mercaptoethanol (1 mM), dithiothreitol (1 mM), or disodium EDTA (1 mM and 10 mM); and the residual activity was determined. The effect of CaCl_2 on hemolysin was determined by the addition of 1 or 10 mM CaCl_2 to the assay mixture. Sterols were dissolved in ethanol to make a 5 mM solution which was serially diluted with hemolysin assay buffer just before use. A small volume of crude hemolysin was added to 1 ml of sterol solution and

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TABLE 1. Extracellular products produced by 120 clinical isolates of *P. cepacia*

Extracellular product ^a	No. of positive strains (%)
Protease	83 (69)
Lecithinase	80 (67)
Lipase	88 (73)
Hemolysin	5 (4)
Elastase	0 (0)

^a Extracellular products were determined by plate assays, as described in the text.

incubated at 0°C for 30 min. Then, the hemolysin activity was determined under standard assay conditions.

Mutagenesis. An overnight culture (4 ml) of *P. cepacia* JN106 was incubated with 12 ml of fresh nutrient broth at 28°C for 4 h with shaking. Cells were harvested by centrifugation, washed once with 0.1 M citrate buffer (pH 5.0), and suspended in 8 ml of the same buffer. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; 2 ml) was added to a final concentration of 1 mg/ml, followed by incubation at 28°C for 90 min without shaking. To screen hemolysin-negative mutants, cells were washed twice with carbon source-free M9 medium (5) and spread on sheep blood-agar plates, which were incubated at 28°C. About 0.5% of the cells remained viable after NTG treatment.

Isolation of plasmid DNA and digestion with restriction enzyme. Plasmid DNA was isolated by the method of Kado and Liu (15). DNA was subjected to 0.7% agarose gel electrophoresis in Tris-borate buffer (89 mM Tris, 2.5 mM EDTA, 89 mM boric acid [pH 8.0]) at a constant 100 V. Gels were stained with ethidium bromide (1 µg/ml) and photographed. Digestion of plasmid DNA with *Bam*HI (Takara Shuzo Co. Ltd., Kyoto, Japan) was performed by the method of the supplier.

RESULTS

Detection of extracellular products. One hundred and twenty clinical isolates of *P. cepacia* were screened for the production of protease, lecithinase, lipase, hemolysin, and elastase (Table 1). The majority of strains produced protease, lecithinase, and lipase. Only 5 strains produced hemolysin, as indicated by a clear zone of hemolysis around the colonies on sheep blood-agar plates. All the hemolysin-producing strains also produced protease, lecithinase, and lipase. None of the strains produced elastase. Among 120 strains, 30 strains did not produce any of the extracellular products described above.

Characterization of hemolysin produced by *P. cepacia* JN106. Because hemolysins produced by various bacteria are known to be involved in bacterial virulence, we characterized the *P. cepacia* hemolysin produced by strain JN106.

TABLE 2. Sensitivity of erythrocytes from various animal species to strain JN106 hemolysin^a

Source of erythrocytes	Relative sensitivity (%)
Human	195
Horse	142
Sheep	100
Chicken	97
Rabbit	77

^a Culture supernatant (8.8 HU/ml) was used.

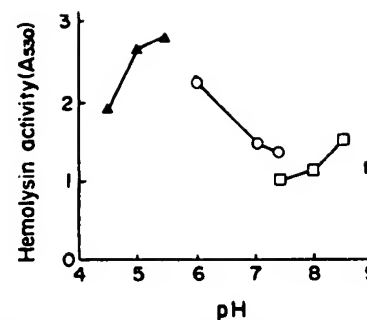


FIG. 1. Effect of pH on hemolysin activity. The culture supernatant of *P. cepacia* JN106 (8.0 HU/ml) was incubated with 2% sheep erythrocyte suspensions in 10 mM buffers of various pHs containing 160 mM NaCl. Symbols: ▲, sodium acetate buffer; ○, sodium phosphate buffer; □, Tris hydrochloride buffer; ■, glycine-NaOH buffer.

When the cells of JN106 were grown in nutrient broth or in dialysate of brain heart infusion broth at 28°C for 48 h without shaking, the culture supernatant contained approximately 8 HU of hemolysin per ml. On the other hand, the activity could not be detected when the cells were grown with shaking but, otherwise, under the same conditions.

About 60% of the hemolysin activity in the culture supernatant was recovered by ammonium sulfate fractionation in the fraction from 20 to 60% saturation. Attempts to purify the hemolysin by gel chromatography or ion-exchange column chromatography were hindered by aggregation of the hemolysin (data not shown).

For the standard assay of hemolysin, sheep erythrocytes were used. In addition to sheep erythrocytes, the hemolysin showed cytolytic activity against human, horse, chicken, and rabbit erythrocytes (Table 2). Human erythrocytes were twofold more sensitive to the hemolysin than were sheep erythrocytes.

The effect of pH on hemolysin activity was determined with the culture supernatant, which showed the highest activity at pH 5.5. This was twice as much as the activity at pH 7.4 (Fig. 1).

Inactivation studies. The hemolysin was heat labile, and 94 and 97% of the activity was lost by heat treatment at 56°C for 30 min or 100°C for 5 min, respectively (Table 3).

The activity of hemolysin against sheep erythrocytes was not affected by *N*-ethylmaleimide, *p*-chloromercuribenzoate, 2-mercaptoethanol, dithiothreitol, EDTA, or CaCl₂. In addition, the hemolysin was not activated by 1 mM dithiothreitol, even after incubation at 4°C for 15 h.

Inhibition of hemolysin by sterols. Cholesterol and 7-dehydrocholesterol completely inhibited hemolysin activity at a concentration of 3 µM, and 50% inhibition was observed on the addition of 0.5 µM of the sterols (Fig. 2). In addition, ergosterol, dihydrocholesterol, and stigmasterol showed 50% inhibition at concentrations of 3 to 5 µM. Concentra-

TABLE 3. Heat stability of strain JN106 hemolysin

Treatment ^a	Residual activity (%)
100°C, 5 min	3.2
100°C, 20 min	1.8
56°C, 30 min	5.6

^a A total of 0.1 ml of crude hemolysin (10 HU) was dissolved in hemolysin assay buffer and incubated under various conditions.

tions of sterols to give 50% inhibition were variable, depending on the hemolysin preparations. No inhibition was observed by 50 μ M dehydroepiandrosteron, pregnenolone, or estradiol (data not shown).

Isolation of hemolysin-negative mutants of JN106. *P. cepacia* JN106 produced protease, lecithinase, and lipase, in addition to hemolysin. To establish that the hemolysin activity was not associated with the other extracellular products, hemolysin-negative mutants were isolated after NTG treatment. By screening 1,500 clones, 4 hemolysin-negative mutants were obtained (Table 4). The mutant JN106 did not produce any hemolysin, as judged by plate or tube assay, by using a sample of the 20 to 60% ammonium sulfate fraction containing 0.25 mg of protein. In contrast, the same ammonium sulfate fraction of the parent strain (crude hemolysin) had approximately 40 HU/mg of protein. Other mutants appeared to be slightly leaky, producing a narrow hemolytic zone on plates after incubation for 4 days at 28°C. Other extracellular products were produced normally by the mutants, except for mutant JN1075, which did not produce protease (Table 4). These results indicate that the hemolysin activity of *P. cepacia* JN106 is not associated with the other extracellular products.

Plasmid analysis of JN106. Strain JN106 was found to carry a plasmid of approximately 58 kilobase pairs, based on the size of restriction fragments produced by digestion with *Bam*HI. All the mutant strains also carried a plasmid of the same size as that carried by the parent strain. It was assumed that the plasmid was not involved in the hemolysin synthesis of JN106.

DISCUSSION

The results of the survey of extracellular products of 120 strains of *P. cepacia* described in this report coincided well with those of McKeivitt and Woods (19), who screened for extracellular products of 48 strains of *P. cepacia* isolated from patients with cystic fibrosis. It appears, therefore, that the described pattern of extracellular products (Table 1) may represent the pattern of *P. cepacia* populations of clinical origin. Gonzalez and Vidaver (11) reported that onion maceration tests and pectolytic activity at low pHs were positive in strains of plant origin, whereas these activities were negative or minimal in strains of clinical origin.

Hemolysin is considered to be one of the virulence factors of several bacteria such as *Streptococcus pyogenes* (1) *Staphylococcus aureus* (14), *Vibrio parahaemolyticus* (21),

TABLE 4. Properties of hemolysin-negative mutants of strain JN106

Strain	Production of ^a :			
	Hemolysin	Protease	Lecithinase	Lipase
JN106 (wild type)	+	+	+	+
JN1066	\pm^b	+	+	+
JN1067	—	+	+	+
JN1070	\pm	+	+	+
JN1075	\pm	—	+	+

^a Extracellular products were determined as described in the text.

^b Slight hemolysis was found after incubation for 4 days.

Vibrio vulnificus (16), *Escherichia coli* (29), and *Pseudomonas aeruginosa* (17). The heat-labile hemolysin produced by *P. aeruginosa* is phospholipase C, which catalyzes the hydrolysis of phosphatidylcholine (lecithin) to phosphorylcholine and diacylglycerol (2). The hemolysin-producing strain *P. cepacia* JN106 also produced lecithinase, as determined by the egg yolk reaction. Association of the hemolytic activity with lecithinase is unlikely, because hemolysin-negative mutants still produced lecithinase.

The hemolysin produced by strain JN106 was inhibited by several sterols. Cholesterol and 7-dehydrocholesterol showed the strongest inhibition of the hemolysin. The inhibition pattern of *P. cepacia* hemolysin by sterols was very similar to that of streptolysin O, a representative of thiol-activated and cholesterol-binding cytotoxins (25). In contrast to streptolysin O, however, the *P. cepacia* hemolysin was not activated by 2-mercaptoethanol or dithiothreitol. Hemolysin produced by *V. vulnificus* is also not affected by 2-mercaptoethanol or dithiothreitol, although it is inhibited by cholesterol (12).

Results of many studies have shown that the genetic determinants for certain virulence factors are carried by plasmids. For example, *E. coli* strains of animal origin carry a hemolysin gene on a plasmid (10). We analyzed strain JN106 and found that it contains a 58-kilobase-pair plasmid. The hemolysin determinant, however, is unlikely to reside on the plasmid, because hemolysin-negative mutants still carry the plasmid.

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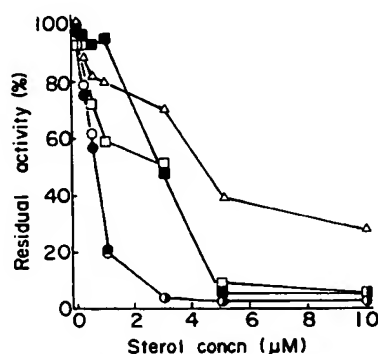


FIG. 2. Effect of sterols on *P. cepacia* hemolysin. Crude hemolysin (1 HU) was incubated with various concentrations of sterols. Symbols: ○, cholesterol; ●, 7-dehydrocholesterol; □, dihydrocholesterol; ■, ergosterol; △, stigmasterol.

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MiniReview

Virulence factors of *Burkholderia cepacia*

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Introduction

Originally named following its identification as a cause of soft rot in onions, *Burkholderia cepacia* has also been known as *Pseudomonas multivorans* and *Pseudomonas kingae* [1,2], and until most recently, as *Pseudomonas cepacia* [3]. A proposal for the transfer of 7 species of the genus *Pseudomonas* RNA Homology group II to a new genus *Burkholderia* with the type-species *Burkholderia cepacia* has been validated [3]. For the purpose of this review the name *B. cepacia* will be used.

Once considered solely as a phytopathogen, *B. cepacia* is now recognized as an important pathogen in nosocomial infection and in patients with chronic granulomatous disease and particularly in those with cystic fibrosis (CF). CF is the most common autosomal recessive lethal disease in Caucasian populations with an incidence of approximately 1 in 2500 live births and a carrier

frequency of 1 in 20. The basic cause of the pathophysiological symptoms of CF is a defect in epithelial ion transport which results in viscous dehydrated bronchopulmonary and gastrointestinal secretions. Build-up of viscid mucus is associated with impaired mucociliary clearance and susceptibility to bacterial colonization which in turn initiates a vicious cycle of chronic inflammatory reaction. The susceptibility of CF patients to pulmonary colonization has been recognised since the earliest descriptions of the disease when patients seldom survived infancy. Advances in management of CF have meant that today most patients survive to early adulthood. However, this increased longevity has in part created its own problems including the emergence of new opportunistic pathogens, including and most notably, *B. cepacia*.

The last decade has seen *B. cepacia* emerge as a particular problem amongst patients with CF, where colonization may be symptomless or associated with a slow decline in lung function. A more serious clinical outcome, not observed with other CF pathogens, in which some colonized CF patients experience *B. cepacia* bacteraemia and/

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or succumb to an accelerated and fatal deterioration in pulmonary function [4-7], is central to the current concern over *B. cepacia* in the CF community.

Based on nucleic acid homology, *B. cepacia* is more closely related to *B. pseudomallei*, *B. mallei* and *B. gladioli* than to *P. aeruginosa* and other fluorescent pseudomonads, and was placed in the separate subgroup, *Pseudomonas* RNA homology group II. *B. cepacia* is nutritionally versatile, with minimal growth requirements and the ability to survive in unfavourable environments: it has been isolated from disinfectants and antiseptics and can even use penicillin G as a nutrient [8-11]. The organism is intrinsically resistant to most antibiotics, and even if individual strains show in vitro susceptibility to an antibiotic, there is little clinical response [10,12-15].

In contrast to the large amount of information on *P. aeruginosa* virulence factors, knowledge of the virulence factors and pathogenesis of *B. cepacia* is scanty. Animal models have indicated that *B. cepacia* is less virulent than *P. aeruginosa* [16]. The aim of this review is to discuss the main features and properties of *B. cepacia* and, in particular, to focus on those which may contribute to its ability to colonize patients with CF.

Colonization and adherence

The ability of a potential pathogen to adhere to the host mucosal or epithelial cell surfaces is often pivotal in the subsequent establishment of infection. Few potential adhesins have been described for *B. cepacia*, and most attention to date has been focused on the adhesive properties of fimbriae.

Electron microscope studies have shown that approximately 60% of *B. cepacia* strains express peritrichous fimbriae (see Fig. 1) [17,58]. Other *B. cepacia* strains possess polar fimbriae, similar to those expressed by *B. aeruginosa* [18]. Kuehn et al. [17] showed that outer membrane protein preparations of *B. cepacia* were enriched with 3 proteins (16, 20 and 40 kDa) which were not present in a non-fimbriated strain. The fimbrial subunit was identified as the 16 kDa protein; the

protein appeared similar to those seen in other bacteria and showed homology with PAK fimbriae of *P. aeruginosa* [17]. This data contrasts with that of Saiman et al. who found minimal cross-reactivity with anti-*P. aeruginosa* anti-pilin monoclonal antibodies and no homology between *P. aeruginosa* pilin gene probes and *B. cepacia* genomic DNA [18,19]. It is possible that sequence variation exists among the pilin genes of different *B. cepacia* strains and that any individual pilin gene probe from *P. aeruginosa* may not reveal a specific *B. cepacia* gene [17].

The presence of fimbriae increases the ability of *B. cepacia* to adhere to pneumocytes in vitro [17]. In vitro binding experiments by Kirvan et al. [20] demonstrated that both *B. cepacia* and *P. aeruginosa* adhere to the same Gal β 1-4GalNAc sequence present in many asialoglycolipids. The experiments of Saiman et al. [19] did not demonstrate competition for epithelial receptors, indicating that different epithelial receptors may be used preferentially by each of the *Pseudomonas* species or that the bacteria may bind to each other. Binding of 2 *B. cepacia* strains to epithelial monolayers increased in the presence of *P. aeruginosa* indicating a possible synergistic relationship whereby *P. aeruginosa* exo-products modify epithelial cell surfaces, exposing receptors and facilitating increased *B. cepacia* attachment [19]. It must be stressed, however, that not all CF patients are colonized with *P. aeruginosa* prior to acquisition of *B. cepacia*: in the Edinburgh CF clinic 38% of patients with *B. cepacia* are not co-colonized with *P. aeruginosa* [6].

Sajjan et al. [21] were able to demonstrate specific binding of *B. cepacia* isolates from patients with CF to both CF and non-CF mucins as well as to buccal epithelial cells. Unfortunately no typing data was available to exclude the possibility of clonal relationships between the strains [21]. The degree of binding observed with *B. cepacia* is considerably less than that observed with *P. aeruginosa* [58]. Deglycosylation of mucin indicated that the mucin receptors for *B. cepacia* include N-acetylglucosamine and N-acetylgalactosamine. Isolates exhibiting the highest mucin binding values tended to correlate with those patients with severe illness leading to speculation

that variability in the binding of different *B. cepacia* isolates to respiratory mucin may contribute to morbidity and mortality, and may explain why some *B. cepacia* strains colonize patients transiently whereas other strains, once acquired, are never lost. A sparsely distributed 22 kDa pilin-associated protein was identified as a mucin binding adhesin specific to piliated strains of *B. cepacia* [22].

Siderophores

Production of siderophores enables bacteria to compete for iron with host iron-binding proteins including transferrin and lactoferrin and has been correlated with the ability of various bacteria to

establish and maintain infection. *B. cepacia* strains express at least 3 siderophore-mediated iron transport systems, including pyochelin, cepabactin and azurechelin [23–26]. Pyochelin produced by *B. cepacia* is chemically unrelated to the pyochelin siderophore of *P. aeruginosa* [23,24]. A 14 kDa ferripyochelin binding protein, present in increased amounts in the outer membrane of iron-starved *B. cepacia* cells, has also been described [23]. Morbidity and mortality in infected CF patients has been correlated to the production of pyochelin. Although such evidence may point to a role in pathogenicity, half of the clinical isolates investigated by Sokol [23] were pyochelin-negative. Pyochelin may increase the ability of *B. cepacia* to disseminate throughout the lungs and perhaps induce a greater inflammatory

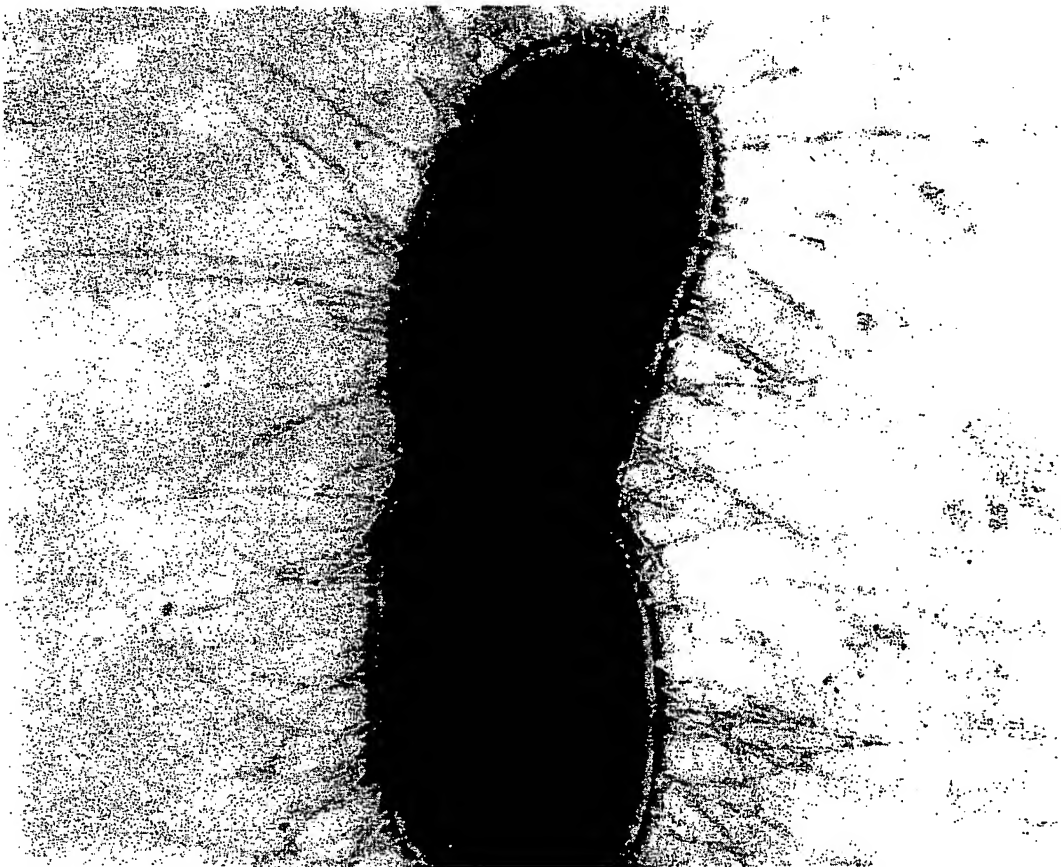


Fig. 1. *Pseudomonas cepacia* J1359 in the process of dividing into 2 separate cells which exhibit peritrichous pili. Staining is 2% w/v phosphotungstic acid. Magnification $\times 25000$.

response due to the increased area of infection in the lung [27]. Exogenously supplied pyochelin enhanced the virulence of non-pyochelin producing *B. cepacia* strains in a chronic pulmonary model in rats [27]. Meyer et al. [26] showed that *B. cepacia* ATCC25416 excreted both pyochelin and a lower molecular mass compound, cepabactin, which strongly chelated Fe III and facilitated iron translocation. Azurechelin, another distinct iron-binding compound, has been identified in 88% of *B. cepacia* strains isolated from the respiratory tract [25].

Extracellular virulence factors

B. cepacia produces a number of extracellular products including protease, gelatinase, haemolysin and lipase, although no pathogenic role for these factors has been demonstrated [28,29]. Unlike *P. aeruginosa*, *B. cepacia* does not appear to produce toxin A, exoenzyme S, or other detectable extracellular factors capable of producing a cytotoxic effect in vitro [28]. In a study of putative pathogenic factors of *B. cepacia* [30] a number of characteristics were demonstrated more frequently in isolates from CF patients than control isolates. These factors included production of catalase, ornithine decarboxylase, valine aminopeptidase, C14 lipase, alginate, and trypsin; reduction of nitrate to nitrite; hydrolysis of urea and xanthine and complete haemolysis on bovine red blood cells. The role of any of these factors in respiratory colonization or infection in CF patients is not clear [30]. Indeed, an epidemic strain of *B. cepacia* CF5610 associated with fatal clinical outcome in CF does not produce C14 lipase or haemolysis [6].

Molecular studies of the *Pseudomonas* exotoxin A gene by Vasil et al. [31] concluded that the production of exotoxin A and the presence of the exotoxin A gene are probably limited to *P. aeruginosa* and is not found in other *Pseudomonas* spp. Southern hybridization experiments under low, medium and high stringency conditions with an exotoxin A gene probe failed to produce a positive signal with any of 8 *B. cepacia* strains tested. Similar experiments con-

ducted by ourselves also failed to demonstrate the presence of the exotoxin A gene in 3 environmental strains of *B. cepacia* although a positive band was obtained with *B. cepacia* CF5610 strain isolated from a patient with CF. However, growth of this strain in both iron replete and depleted medium and subsequent analysis of the cell free culture supernate by polyacrylamide gel electrophoresis and immunoblot analysis with anti-*P. aeruginosa* exotoxin A antisera, failed to confirm production of a 66 kDa protein equivalent to *P. aeruginosa* toxin A. (J.W. Nelson, unpublished results).

The extracellular proteinase of *B. cepacia*, a 34 kDa protein, has antigenic similarities to *P. aeruginosa* elastase and cleaves gelatin, hide powder, collagen but not human immunoglobulin IgG, IgM, secretory IgA, or IgA [32]. Intratracheal instillation of purified proteinase into rat lungs produces a bronchopneumonia characterized by polymorphonuclear cell infiltration and proteinaceous exudation into large airways. Active immunization of rats with *B. cepacia* proteinase elicits an immunological response although this is not protective against subsequent lung infection with *B. cepacia* [32].

There is also evidence that lipases, particularly phospholipases, may play an important role in bacterial virulence [33,34]. Phospholipase C is an enzyme that cleaves phosphatidylcholine, a major lung surfactant, to yield phosphorylcholine and diacylglycerol, and has been associated with cytopathology of lung tissue. *B. cepacia* has frequently been described as being lipolytic [28,33-35]. McKevitt and Woods [28] reported that 32 of 48 strains of *B. cepacia* isolated from CF patients demonstrated lipase activity on egg-yolk agar whilst Carson et al. [35] showed that *B. cepacia* could hydrolyse Tween 20, 40, and 80. In another study [34] 6 out of 10 clinical strains of *B. cepacia* from the sputum of CF patients produced lecithinase by the egg-yolk reaction, whilst lipase activity on 4 different Tweens was strain-dependent. Purified enzyme had a molecular weight of 25 000 and was not cytotoxic for Hela cells or for mice injected intravenously with purified lipase. It has been reported, however, that lipase adversely affected the phagocytic function of rat pulmonary

alveolar macrophages in a dose-dependent manner [36]. Phagocytosis of *B. cepacia* by rat pulmonary alveolar macrophages was significantly reduced when the cells were either preincubated with lipase or when phagocytosis occurred in the presence of the lipase [36]. Scanning electron microscopy showed that the macrophages exposed to *B. cepacia* lipase had fewer pseudopodia, microvilli and other projections compared to untreated macrophages. Thus *B. cepacia* lipase may be an important virulence factor which allows the bacteria to evade the mammalian host defence system.

B. cepacia produces a heat-labile haemolysin which has both phospholipase C and sphingomyelinase activities [37]. Haemolytic and phospholipase C (lecithinase) expression in *B. cepacia* appears to be a complex phenomena. The study of Nakazawa et al. [29] found that only 4% of clinical isolates were β -haemolytic, whilst 67% of isolates produced lecithinase. Others have found higher percentages of haemolytic isolates of *B. cepacia* if a variety of erythrocyte types were tested, including a study of clinical isolates of *B. cepacia* from CF patients which found that 40% were haemolytic when erythrocytes from various animals were tested [37]. Unlike the PLC activity of *P. aeruginosa* the PLC activity in *B. cepacia* does not correlate with haemolytic activity [29,37]. However, all haemolytic strains produce detectable lecithinase activity, and strains of *B. cepacia*, whether haemolytic or non-haemolytic, appear to produce detectable amounts of extracellular PLC activity. In contrast to the consistent patterns observed in the PLC gene of *P. aeruginosa* there is hypervariability in genetic organization of the PLC gene of *B. cepacia* [37]. The variable manner in which a *B. cepacia* PLC specific gene probe hybridizes with restricted *B. cepacia* DNA, the variability in expression of haemolytic and PLC activities of different strains, and the association of DNA arrangements with conversion of an Hly+ to an Hly- variant may be related to the relatively large number of distinct insertion sequences (IS) reported for *B. cepacia* (> 25) [37,38]: in contrast these elements have yet to be discovered in *P. aeruginosa*. Some of these IS elements of *B. cepacia*, can be found

in multiple copies and have been shown to both activate or inactivate gene expression.

Cell surface antigens

Lipopolysaccharide

B. cepacia strains isolated from patients with CF may express either the rough (R) or smooth (S) lipopolysaccharide (LPS) phenotype, whereas the majority of *B. cepacia* strains isolated from other clinical conditions or from the environment express S-LPS (S.L. Butler, unpublished results). This is in agreement with the study of McKevitt and Woods [28] where 22 strains examined possessed S-LPS and 26 strains possessed R-LPS. The epidemic strain of *B. cepacia* isolated from a number of CF patients in the UK invariably has a R-LPS phenotype and is associated with the appearance of dry colonies [6]. There is no evidence to date to confirm that *B. cepacia* strains undergo a phenotypic change from S to R LPS within the CF lung as is observed with *P. aeruginosa*.

Western blotting and absorption studies demonstrated that a significant proportion of serum antibodies from *B. cepacia*-infected CF patients which reacted with the core LPS of *B. cepacia* did not react with the core LPS of *P. aeruginosa* [39]. These observations indicate differences in the structure and composition of core LPS between *B. cepacia* and *P. aeruginosa* confirming previous findings, including the lack of phosphorus in the core of *B. cepacia* LPS [40] and the inability of a monoclonal antibody reactive with *P. aeruginosa* and *P. fluorescens* core LPS to react with *B. cepacia* [41]. Core heterogeneity between different isolates of *B. cepacia* may also exist because immunoblotting demonstrated that serum from patients colonized with *B. cepacia* produced a band reactive with some but not all core LPS preparations [39].

Initial chemical analysis of *B. cepacia* LPS indicated the absence of detectable 3-deoxy-D-manno-2-octulosonic acid (KDO) in LPS from *B. cepacia* [42,43]. However, Straus et al. [44] reported the isolation of KDO from the culture supernate of 2 out of 10 strains of *B. cepacia* and

in a further study KDO was demonstrated in 6 clinical isolates of *B. cepacia* and all 6 LPS preparations were equally toxic for mice when injected intraperitoneally [45]. Compared with LPS from *P. aeruginosa* that from strains of *B. cepacia* has less phosphorus and more heptose. Glucose and rhamnose were the major saccharide components of LPS from the organisms tested [42]. An extracellular material isolated from a clinical *B. cepacia* consisted of a surface carbohydrate antigen, LPS and protein, the toxicity of which appeared to be associated with the LPS portion of the complex [46]. It has been proposed that this extracellular toxic complex produced by *B. cepacia* is responsible for the lethality and extensive pulmonary tissue necrosis associated with pneumonia produced by this organism.

Outer membrane proteins

B. cepacia produces 5 major outer membrane proteins A (56 kDa), B (38 kDa), C (37 kDa), D (28 kDa) and E (21 kDa). The C and D proteins have been identified as porin proteins [47,48], and appear to be antigenic in most patients with CF who are chronically colonized with *B. cepacia* [48,49]. In the study of Anwar et al. [55], outer membrane protein profiles of magnesium-depleted cells were much simpler than that of iron-depleted cells and nutrient broth grown cells. Synthesis of a 66 kDa outer membrane protein was induced when *B. cepacia* was grown under iron depletion. *B. cepacia* isolates from individual CF patients may exhibit marked phenotypic variability, including manifestation of different patterns of outer membrane proteins separated on a polyacrylamide gel: up to 5 OMP patterns have been identified from *B. cepacia* isolates derived from a single strain [50].

Various studies indicate that the outer membrane of *B. cepacia* is a major contributing factor in the β -lactam resistance of this species, retarding the diffusion of β -lactams to their penicillin-binding protein targets [47,51,52]. Resistance to aminoglycosides and hydrophobic compounds in *B. cepacia* is largely due to the low outer membrane permeability [51]. Loss of the major porin protein D and decreased expression of protein C

may also be associated with high level β -lactam resistance in some CF isolates of *B. cepacia* [52]. Production of β -lactamases, including carbapenemases capable of hydrolyzing the most potent and broad spectrum of the β -lactam antibiotics, imipenem and meropenem, also contribute significantly to the resistance of *B. cepacia* [53].

Exopolysaccharide

Production of alginate by mucoid strains of *P. aeruginosa* is the major virulence determinant associated with strains which colonize the lungs of patients with CF. In contrast, *B. cepacia* does not appear to produce alginate. PCR studies with primers of the *P. aeruginosa* *algD* gene, encoding the essential enzyme GDP mannose dehydrogenase, indicate that this gene was absent in 10 *B. cepacia* strains studied and therefore that *B. cepacia* is unlikely to produce an alginate-like polymer (J.W. Nelson, unpublished results). Additional studies in our laboratory and by Sage et al. [54] showed that some *B. cepacia* strains do produce an exopolysaccharide comprising galactose, glucose, mannose, glucuronic acid and rhamnose, with lesser amounts of uronic acid: no mannuronic or guluronic acid was detected. Surveys of clinical isolates from patients with CF indicate that there is no correlation between the ability of *B. cepacia* to colonize the respiratory tract and capacity to form exopolysaccharide [6,54]. In contrast, Straus et al. [46] observed that 1 strain of *B. cepacia* produced an alginate-like compound containing 72% guluronic acid with 1.75% acetylation.

Evasion of the immune system

Immunological studies on *B. cepacia* colonization of patients with CF indicate that the organism persists despite a considerable antibody response and suggest the possibility of immune-mediated damage. Aronoff et al. [48,49] demonstrated the presence of IgG antibodies to outer membrane antigens of *B. cepacia* in serum from patients with CF colonized with *B. cepacia* and/

or *P. aeruginosa*. These authors concluded that some *B. cepacia* outer membrane components may be antigenically related to those of *P. aeruginosa* and that colonization with *B. cepacia* occurs in the presence of antibodies specific for the outer membrane of the organism. Serum IgG and sputum IgA antibodies directed towards the core LPS of *B. cepacia* have also been described [39].

Investigations into the bactericidal effect of human serum have shown a large variation in the responses of the *B. cepacia* strains investigated. All strains expressing R-LPS were serum-sensitive under a variety of test conditions whilst strains expressing S-LPS exhibit a range of responses (S.L. Butler, unpublished results). Anwar et al. [55] showed that a *B. cepacia* strain grown in different nutrient depletions in batch culture showed varying degrees of sensitivity to engulfment and killing by human polymorphonuclear leucocytes (PMN) and to killing by human serum. The wide range of sensitivity shown by the organism may reflect the phenotypic variation in cell envelope composition caused by specific nutrient depletions. Patients with chronic granulomatous disease (GCD) are at particular risk of infection with *B. cepacia*, which is able to resist neutrophil-mediated non-oxidative bactericidal killing (D.P. Speert, personal communication). The ability of *B. cepacia* to survive a pronounced humoral response and other immunological defences is intriguing and requires further investigation. Indeed there is a suggestion that *B. cepacia* may invade and survive within respiratory epithelial cells, enabling the organism to persist within the CF lung [56].

Concluding remarks

Acquisition of *B. cepacia* is a major concern among patients with CF although the exact pathophysiological role of the organism remains controversial and unsolved. Epidemiological data and the use of phenotypic and genotypic typing systems for *B. cepacia* suggest that certain strains are particularly transmissible, although there is no evidence at present that some strains are more virulent. The role of any of the described viru-

lence factors of *B. cepacia* relating to its pathogenesis in patients with CF remains unclear. Enhanced adhesion to mucin of certain *B. cepacia* strains may aid initial colonization whilst multi-resistance to antibiotics and possible intracellular localization may contribute to persistence of the organism. Production of anti-*B. cepacia* antibodies by the host and subsequent immune complex mediated damage, is probably responsible for pulmonary decline. The development of a CF mouse model carrying precise and clinically relevant mutations [57] will allow in vivo investigation of *B. cepacia* colonisation and virulence factors. Current concern over *B. cepacia* colonisation amongst CF patients has highlighted the urgent need to identify *B. cepacia* colonising factors and the pathophysiological and/or immunological factors which account for the rapid clinical deterioration in some CF patients.

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Soft-Tissue Infection Due to *Mycobacterium smegmatis*: Report of Two Cases

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Mycobacterium smegmatis is an uncommon pathogen in humans. Fourteen cases of skin or soft-tissue infection due to *M. smegmatis* have been previously reported. We report two cases of posttraumatic *M. smegmatis* infection of the lower extremity. *M. smegmatis* infection produces chronic cellulitis with fistula formation that is most commonly a result of direct traumatic inoculation of contaminated material. Extensive surgical debridement followed by skin grafting has been necessary for cure in the majority of cases.

Mycobacterium smegmatis, a rapidly growing mycobacterium, is an uncommon cause of disease in humans. The first well-described case involving a human was that of a patient with *M. smegmatis* pleuropulmonary infection and was reported by Vonmoos et al. [1] in 1986. Since then, the cases of 25 additional patients with *M. smegmatis* infection, 14 of which had skin or soft-tissue infection, have been reported [2-4]. Among the rapidly growing mycobacteria, *M. smegmatis* is now considered second only to the *Mycobacterium fortuitum* complex as a cause of human disease [5].

Case Reports

Patient 1. A 21-year-old man was involved in an accident during which he was ejected from a motor vehicle and sustained closed fractures of the left scapula and the right first rib as well as a small puncture wound of the distal left leg. The puncture wound was irrigated and cleaned of debris on the day of injury. Six weeks later the patient presented to the orthopedic clinic at our facility (Naval Hospital, San Diego) with complaints of draining of the left-leg lesion of 4 weeks' duration and painful left inguinal lymphadenopathy of 3 days' duration. The patient denied experiencing fever, chills, night sweats, or weight loss.

Physical examination on admission revealed a temperature of 36.8°C and a normal pulse and blood pressure. A warm, erythematous area that measured 9 × 9 cm was present on the lateral aspect of the distal left leg and ankle.

Within this area was a fluctuant mass (1.5 × 2 cm) with central ulceration and seropurulent drainage. Tender, non-fluctuant left inguinal lymphadenopathy with lymphangitic streaking was also present. Radiographs of the distal left tibia revealed periosteal elevation, and a radionuclide bone scan showed intense focal uptake in the medial portion of the left tibia and in the surrounding soft tissue.

The patient underwent operative debridement, and extensive soft-tissue and periosteal necrosis was noted. Gram staining of the debrided tissue revealed many polymorphonuclear leukocytes but no organisms. Ziehl-Neelsen staining was not performed. The patient's wound healed normally, and on the 9th postoperative day acid-fast bacilli were noted in cultures of specimens obtained during the operative debridement. Therapy with cefoxitin (2 g iv every 8 hours) and amikacin (640 mg iv every 12 hours) was begun empirically. Disk diffusion testing of the bacilli showed marked sensitivity to trimethoprim-sulfamethoxazole (TMP-SMZ); consequently, the antibiotic regimen was changed to 1 tablet of TMP-SMZ three times a day. A follow-up visit at 8 weeks showed resolution of the cellulitis with a healed surgical wound, and administration of antibiotics was discontinued. Clinical resolution of the tibial osteomyelitis was also noted, and the patient was still well 7 months after discontinuation of antibiotic therapy. The cultured organism was later identified as *M. smegmatis*.

Patient 2. A 29-year-old severely obese woman was involved in a motor vehicle accident and sustained an avulsion injury (25 × 30 cm) of the anterior left thigh, an open laceration of the right prepatellar bursa, and a traumatic left knee arthrotomy. She was treated at a local hospital, where large amounts of sand and gravel were removed from the wound at initial debridement. Administration of ampicillin, gentamicin, and clindamycin was started empirically. Three days later the patient was transferred to our facility; upon arrival she was febrile (temperature, 38.8°C), tachycardiac (heart rate, 118), and hypotensive (blood pressure, 88/52 mm Hg). Initial examination revealed that the large avulsion injury contained small areas of necrotic tissue and foreign debris; debridement was repeated. *Pseudomonas aeruginosa* grew in cultures of the intraoperative specimens, and antibiotic ther-

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apy was changed to administration of ticarcillin/clavulanate (3.1 g iv every 4 hours). Thereafter, the wounded area progressively improved. Split-thickness grafting of skin was performed 18 days after the third debridement, and the patient was discharged 2 weeks later.

One month after discharge the patient developed cellulitis of the posterolateral aspect of her left thigh, which was empirically treated with dicloxacillin (500 mg po four times a day). Painful, fluctuant nodules developed within the area of cellulitis, and open drainage was performed. Gram staining revealed many polymorphonuclear leukocytes but no organisms. Routine cultures were negative. Acid-fast staining of the debrided tissue was not performed. After 4 months of persistent cellulitis, the patient was readmitted for further evaluation. Examination revealed an erythematous, warm, brawny area of cellulitis (15 × 25 cm) in the left popliteal fossa and posterolateral thigh. Multiple well-formed sinus tracts draining purulent material were present. A radionuclide bone scan and an indium-labeled leukocyte scan showed intense uptake in the medial femoral condyle that was suggestive of osteomyelitis, but a magnetic resonance imaging scan revealed no abnormalities. Ultrasonography revealed an abscess cavity measuring 2.4 × 2 cm within the area of cellulitis, which yielded 8 mL of purulent fluid on needle aspiration. Gram staining of the fluid showed many polymorphonuclear leukocytes with several beaded gram-positive bacilli. Modified Kinyoun carbolfuchsin staining was strongly positive for acid-fast bacilli. The organism was presumptively identified as a *Nocardia* species; the patient was discharged and continued therapy with TMP-SMZ (1 tablet three times a day).

One month later, the patient continued to have pain in and drainage from the thigh lesion. The organism originally isolated had meanwhile been identified as *M. smegmatis*. Antibiotic therapy was changed to the administration of doxycycline (100 mg po twice a day) and ciprofloxacin (750 mg po twice a day) on the basis of sensitivities demonstrated by disk diffusion. The patient continued to have seropurulent drainage from the sinus tracts and required surgical debridement. Extensive necrosis of the subcutaneous tissue that extended to the muscular fascia was noted during surgery. At the time of this writing, the patient was receiving maintenance therapy with ciprofloxacin and doxycycline and was being observed for clinical evidence of recurrent infection.

Discussion

M. smegmatis was isolated initially from syphilitic chancres in 1884 by Lustgarten [6] and, 1 year later, from normal human genital secretions (smegma) by Alvarez and Tavel [7]. This organism has subsequently been recognized as an environmental saprophyte; Tsukamura [8] identified *M. smegmatis* in 25 (38%) of 66 Japanese soil samples. It is interesting that *M. smegmatis* has not been isolated from ei-

ther normal or pathogenic human genital secretions since its initial isolation by Alvarez and Tavel in 1885 [2].

M. smegmatis, a rapidly growing mycobacterium, is microbiologically similar to *M. fortuitum* in that it grows on MacConkey medium without crystal violet, reduces nitrate, and demonstrates iron uptake. However, it can be differentiated from *M. fortuitum* by its growth at 45°C, the negativity of a 3-day test for arylsulfatase, and the delayed formation of pigment at 2 weeks (in 50% of isolates).

As late as 1979, *M. smegmatis* was felt to be nonpathogenic in humans [9]. The first human infection was reported in 1986 by Vonmoos et al. [1], who described a patient with *M. smegmatis* pleuropulmonary infection. The first report of cutaneous disease was published in 1988 by Wallace et al. [2], who reported that 22 clinical isolates had been referred to their lab over a 7-year period. Eleven of these isolates were from patients with cutaneous or soft-tissue infection. A brief description of the patient's medical history accompanied each isolate, but details on the clinical course or response to therapy were not provided.

Plaus and Hermann [3] subsequently reported two cases of soft-tissue infection due to *M. smegmatis* in 1991. Both patients abused anabolic steroids and developed anterior thigh abscesses at the injection site after using a common bottle of veterinary-grade stanozolol that was contaminated with *M. smegmatis*. The patients were treated with erythromycin and amikacin for 2 weeks and then with ciprofloxacin for an additional 6 weeks, but their conditions failed to improve; aggressive debridement followed by skin grafting was required. This procedure resulted in apparent eradication of the infection, as both patients were clinically disease-free 6 months after debridement.

An additional case of cutaneous infection was reported by Roger et al. [4] in 1991. This patient sustained a puncture injury to the ankle while gardening and developed extensive cellulitis with suppuration. The patient was treated with various antibiotic combinations to which the isolate was sensitive in vitro, but failed to respond and required aggressive surgical debridement with subsequent skin grafting.

In summary, *M. smegmatis* is a rare but clinically significant environmental pathogen and should be considered in the diagnosis of chronic cutaneous or soft-tissue infection. A history of a soil-contaminated wound should raise the clinical suspicion of infection with this pathogen. Extensive surgical debridement followed by skin grafting has been necessary for the eradication of infection for most patients. Adjunctive antibiotic therapy should be considered and can be chosen on the basis of in vitro sensitivity data [10].

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Evidence for Nosocomial Transmission of *Candida albicans* Obtained by Ca3 Fingerprinting

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The moderately repetitive sequence Ca3 was used to fingerprint *Candida albicans* isolates from 32 patients hospitalized for more than 3 days, 17 recent admissions or outpatients, and 8 recently readmitted patients and 10 commensal isolates from the community in Wellington, New Zealand, plus isolates from 21 hospitalized patients, 26 outpatients or recent admissions, 4 recently readmitted patients, and 10 healthy individuals in the community in Auckland, New Zealand. In Wellington, isolates from patients hospitalized in Wellington Hospital for more than 3 days were genetically significantly less diverse than were isolates from outpatients or recent admissions or isolates from healthy individuals in the community. In addition, two clusters of genetically similar strains were isolated from hospitalized patients significantly more often than from other individuals. These observations provide evidence (albeit indirectly) for nosocomial transmission of hospital-specific *C. albicans* strains. In contrast, no indication of hospital-specific transmission of *C. albicans* was found in Auckland Hospital. Since these results were obtained under conditions in which no candidiasis outbreak occurred in either hospital, they also suggest that Ca3 fingerprinting may be a useful tool in preventive nosocomial infection control programs, allowing assessment of the extent of *C. albicans* transmission occurring in a hospital.

Most humans carry the yeast *Candida albicans* as part of their commensal microflora, but in hosts predisposed to candidiasis it can act as a pathogen. In hospitals, greater success in the treatment of cancers and in the treatment of viral and bacterial infections and improvement in intensive care have led to an ever-increasing number of severely compromised patients susceptible to candidiasis. This has led to an increase in the incidence of nosocomial candidiasis, with *C. albicans* being the most frequently encountered species. In most patients, candidiasis is localized, causing patients a considerable degree of discomfort. For certain high-risk groups of patients, such as burn patients, low-birth-weight babies, and leukemics, a 5 to 15% incidence of *Candida* fungemia with an associated mortality rate of up to 90% has been reported (1, 4, 6, 7, 22).

It is a commonly expressed opinion that strains which were already present as part of a host's commensal microflora are usually the etiological agents of candidiasis (7, 16, 20). On the other hand, clusters of nosocomial candidiasis do occur, suggesting the possibility of transmission of virulent strains to patients (5). It is important to determine whether transmission contributes to nosocomial candidiasis. If it does, prevention of transmission may reduce the incidence of the disease.

The numerous attempts to demonstrate nosocomial transmission of *C. albicans* have been recently reviewed by Hunter (5). Some of these studies have indeed found the same type of strain on multiple patients but suffered from problems regarding the discriminatory power and reproducibility of the typing methods used. These problems are exemplified by the results of multiple studies of the so-called "London outbreak." As

summarized by Hunter, repeat analyses of this outbreak yielded contradictory results not only when different typing methods were used but also when the same method (restriction fragment length polymorphism on ethidium bromide-stained gels of whole DNA digests) was used twice for the same isolates (5). In his 1991 review, Hunter concluded that nosocomial transmission of *C. albicans* had not yet been unequivocally demonstrated (5). Our own review of the literature indicates that to date this conclusion remains valid.

The present study employed computer-assisted Ca3 DNA fingerprinting (15), the most highly discriminating and accurate *C. albicans* typing method currently available (5, 8), to test if strain transmission between hospitalized patients occurs. Use was made of the ability of the method to quantitatively describe the relationships between groups of isolates on the basis of their genetic similarity (15). By this approach, nosocomial transmission should be revealed by the widespread occurrence of groups of genetically similar strains on hospitalized patients. These groups of strains should in addition be significantly less frequent on outpatients or recent admissions and healthy individuals in the community.

Using this approach, we have obtained evidence for transmission of *C. albicans* in one of two hospitals studied.

MATERIALS AND METHODS

Selection and isolation of strains. Clinical isolates were from patients at Wellington Hospital, Wellington, New Zealand, admitted to 16 different wards and from patients at Auckland Hospital, Auckland, New Zealand, admitted to 27 different wards. Both are tertiary-care hospitals of approximately the same size (546 and 687 beds, respectively). Isolates (one per patient) for fingerprinting were chosen from isolates recovered from clinical specimens submitted to hospital microbiological laboratories between August 1992 and November 1993. All isolates had been identified as *C. albicans* by the germ tube assay (7). This identification was verified by the results of fingerprinting with the species-specific probe Ca3. All isolates had the strong and complex banding patterns characteristic of *C. albicans* but absent in related species (9, 13, 18, 19). All isolates

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fingerprinted were from sites at which yeast concentrations exceeded the levels for commensal colonization listed by Odds (7). Yeast concentrations were determined by semiquantitative plate-streaking procedures employed by the laboratories. These results were converted to cell numbers by using conversion factors derived from calibration experiments with samples of known viable-cell concentrations. Vaginal isolates were excluded from the analysis because earlier studies have indicated that relationships among these isolates differ from those among isolates found in other body locations and that they must thus be assessed separately (11, 14). Commensal isolates (each from a different person) were obtained from nonrelated healthy volunteers in the community. For each isolate, a single colony was used to inoculate an agar slant which formed the basis of further analysis. Additional information on the histories of the isolates is summarized in Table 1 and in Fig. 1 and 5.

DNA fingerprinting with the Ca3 probe. DNA fingerprinting was performed as previously described by Schmid et al. (15). In brief, cells were grown in YPD medium to late log or early stationary phase and DNA was prepared from spheroplasts according to the method of Scherer and Stevens (10). The DNA concentration was determined fluorometrically by using the dye H 33258 (3). DNA was digested with *EcoRI*, after which fragments were separated on 0.8% agarose gels and transferred to a nitrocellulose membrane and then subjected to Southern hybridization with a ³²P-labelled nick-translated Ca3 probe (15) and autoradiography. The Ca3 probe contains 11 kb of repetitive sequences and hybridizes to all but two chromosomes of *C. albicans* (2). For a detailed description of its characteristics as a fingerprinting tool, see reference 15.

Analysis of fingerprints for the determination of relationships between strains. The methods used for analysis of fingerprints were those described by Schmid et al. (15). Southern blot patterns were entered into a Macintosh Ilii computer as data files. The Dendron computer program (15) was used to quantify the degree of similarity between patterns of different isolates, resulting in similarity values, also referred to as S_{AB} values, ranging from 1.0 (the two patterns compared are identical) to 0.0 (the two patterns have no bands in common) (15). Relationships between isolates were visualized in dendrograms constructed from matrices of similarity values by using the unweighted pair group method (17). The branching point between two isolates in dendrograms reflects their genetic similarity. Groups of genetically similar strains were defined as groups of isolates connected by branching points in dendrograms at an S_{AB} value of ≥ 0.8 ; this value lies halfway between the average S_{AB} value found for multiple isolates from the same patients (0.96) (reference 13 and this study) and the average S_{AB} value for isolates from different unrelated healthy individuals in the community (0.66) (references 13, 15, and 18 and this study).

Test for the preferred association of groups of genetically similar strains with patients with particular characteristics. The frequency of isolation of a group of genetically similar strains (defined as described above) from patients showing a particular characteristic (e.g., a particular sex or age, long hospitalization, or admission to a particular ward) was determined. Next, the frequency of the same group of strains among other patients lacking this characteristic (or having the opposite characteristic) was determined. A z test was used to determine if the difference in frequency was statistically significant.

RESULTS

Analysis of Wellington isolates. Sixty-seven *C. albicans* isolates, each from a different individual, were analyzed (Fig. 1). These included 10 commensal isolates (labelled C in Fig. 1) and 57 patient isolates. Of the 57 patients, 32 had been hospitalized for more than 3 days prior to the isolation of *C. albicans* and were categorized by us for the purpose of this study as hospitalized (labelled H in Fig. 1). Seventeen were either outpatients or had been hospitalized for 3 days or less and were categorized by us as outpatients or recent admissions (labelled OR in Fig. 1). Eight additional patients were recent admissions but had been hospitalized within the 6 preceding months and were for that reason not included in the outpatient-or-recent-admission category).

Figure 1 shows that many of the strains were genetically dissimilar. However, several groups of similar isolates are also apparent (such groups being defined as containing isolates with S_{AB} values of ≥ 0.8 between them). The three largest groups of genetically similar isolates are labelled WA, WB, and WC in Fig. 1. Several of the isolates within these groups were as, or almost as, similar to each other as were multiple isolates from the same patients (average S_{AB} value = 0.96 ± 0.02 , determined with two isolates each from four patients). Figure 2A gives an overview of the range of Ca3 patterns encountered; Fig. 2B illustrates the similarities between isolates from the

TABLE 1. Histories of *C. albicans* isolates used in this study

Isolate or patient category	No. (%) of isolates	
	Wellington	Auckland
Patient isolates		
No. of isolates	57 (100)	51 (100)
Sex of patients		
Female	23 (40)	21 (41)
Male	34 (60)	30 (59)
Age of patients (yr)		
0-2	3 (5)	4 (8)
2-50	17 (30)	16 (31)
>50	37 (65)	31 (61)
Most frequent sites of <i>C. albicans</i> isolation		
Sputum or aspirate	16 (28)	18 (35)
Skin or wounds	14 (24)	11 (22)
Feces	11 (19)	0 (0)
Urine	0 (0)	9 (18)
Oral cavity	10 (17)	7 (14)
Most frequent conditions predisposing patient to candidiasis ^a		
Antibacterial antibiotics	13 (23)	12 (24)
Surgery	11 (20)	5 (10)
Catheterization	4 (7)	6 (12)
Asthma (corticosteroid treatment)	10 (17)	1 (2)
Diabetes	0 (0)	3 (6)
No. of patients at high risk of acquiring candidiasis ^b	9 (15)	8 (16)
No. of patients with clinical features of candidiasis reported	32 (56)	17 (33)
Duration of hospitalization of patient prior to isolation of <i>C. albicans</i> (days)		
>3 ^c	32 (56)	21 (41)
0-3 ^d	17 (30)	26 (51)
0-3 readmission ^e	8 (14)	4 (8)
Commensal isolates		
No. of isolates	10	10
Sex of patients		
Female	6	4
Male	4	3
Unknown	0	3
Age of patients (yr)		
0-2	0	0
2-50	9	6
>50	1	1
Unknown	0	3
Site of <i>C. albicans</i> isolation	Oral cavity	Oral cavity

^a Conditions listed by Odds (7) as predisposing patients to candidiasis.

^b HIV-positive or leukemia patients or those undergoing anticancer therapy.

^c The average durations of hospitalization were 32 ± 65 days in Wellington Hospital and 29 ± 26 days in Auckland Hospital.

^d Outpatients and recent admissions. The average duration of hospitalization was 1 ± 1 day in both hospitals.

^e Recent admissions who had been hospitalized within the previous 6 months.

same patient and isolates from different patients falling into the same group of genetic similarity.

If transmission of *C. albicans* between hospitalized patients occurs, isolates from such patients should be derived from a limited number of groups or clusters whose members are sim-

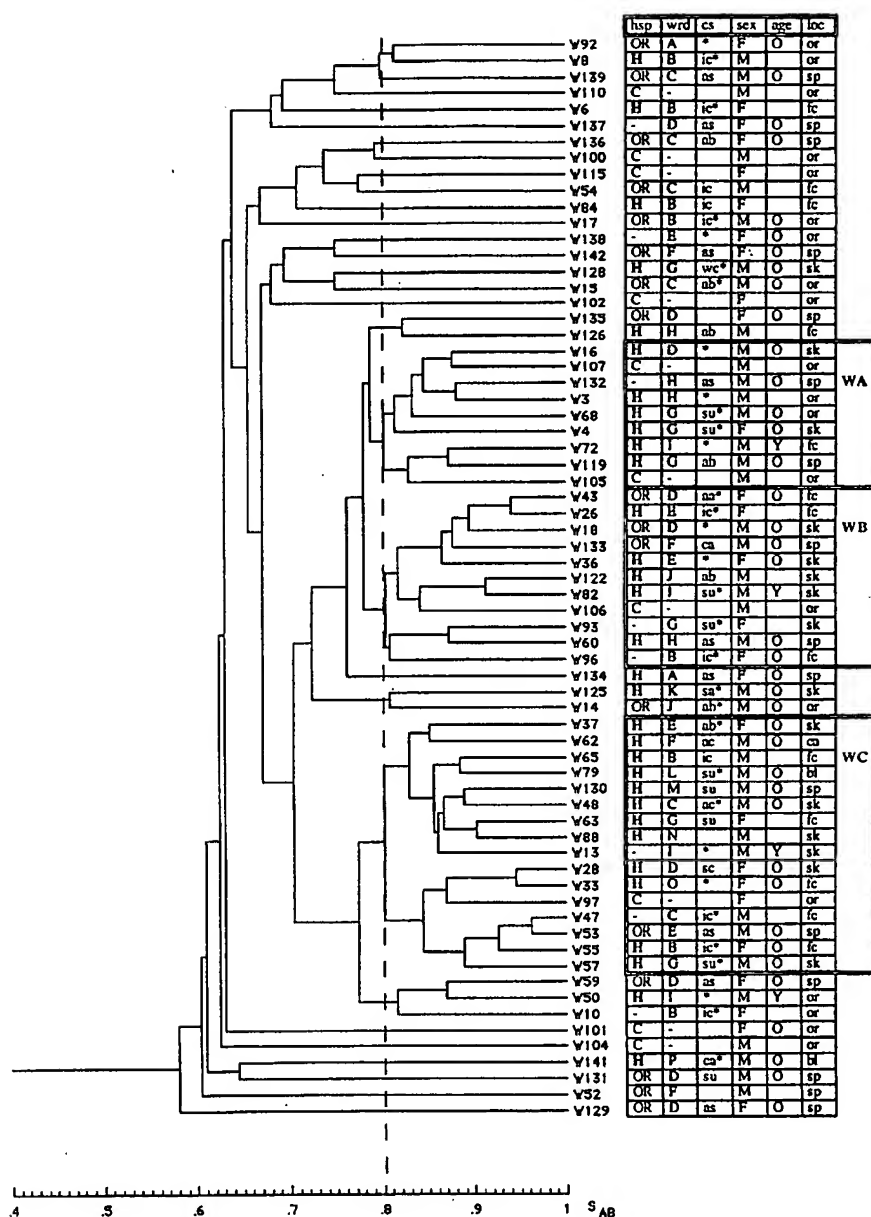


FIG. 1. Genetic relationships among all Wellington isolates determined on the basis of the similarity of their Ca3 fingerprints. The dashed line in the dendrogram denotes the threshold S_{AB} value of 0.8 for defining groups of genetic similarity. The three largest groups of genetically similar isolates, WA, WB, and WC, are marked on the right of the dendrogram. Following the designation of each isolate is information on the duration of hospitalization of the patient (column labelled hsp; H, hospitalized for >3 days; OR, outpatient or recent admission; -, recent admission who was hospitalized previously; C, individual in the community [commensal isolate]), the wards to which patients had been admitted (column labelled wrd), clinical status indicating conditions predisposing the patient to candidiasis (column labelled cs; aa, asthma plus antibiotics; ab, antibiotics; ac, antibiotics plus catheterization; as, asthma; ca, catheterization; ic, immunocompromised by HIV, leukemia, or cancer or cancer treatments; sa, surgery plus antibiotics; su, surgery; wc, wound plus catheter). Asterisks denote patients for whom clinical symptoms indicating candidiasis were reported), sex, age (O, >50 years; Y, <2 years; blank cells, 2 to 50 years), and the body site or material from which the isolate was obtained (column labelled loc; bl, bloodstream; ca, catheter; fc, feces; or, oral cavity; sk, skin, including wounds; sp, sputum).

ilar to each other. In contrast, isolates from recently admitted patients, outpatients, or healthy individuals in the community should be genetically more diverse. Figure 1 indicates that many of the isolates from hospitalized (>3 days) patients in Wellington Hospital were derived from only a few groups, since a high percentage of them were associated with the three large clusters WA, WB, and WC.

To directly assess if a difference in genetic diversity existed

between isolates from hospitalized patients and isolates from outpatients or recent admissions or commensal isolates, we determined the relationships between isolates in each category separately (Fig. 3). Isolates from hospitalized patients were indeed genetically less diverse than were isolates from the other two categories: in dendrograms (Fig. 3A), more than 75% of isolates from hospitalized patients formed clusters with other similar isolates from such patients. In contrast, only 24%

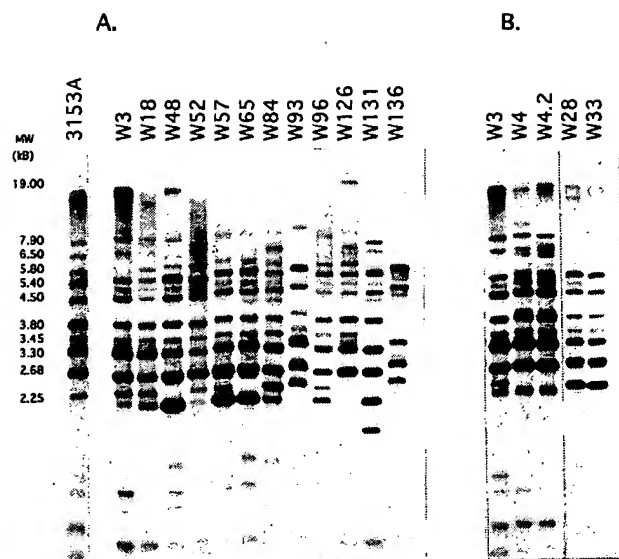


FIG. 2. (A) Range of DNA fingerprints of isolates from different individuals in Wellington plus strain 3153A, which is included as a standard, and (B) examples of fingerprints of isolates from the same groups of genetic similarity (W3 and W4 from group WA and W28 and W33 from group WC) in comparison with fingerprints of multiple isolates from the same patient (W4 and W4.2, isolated on the same day from a wound and a drain site, respectively). Molecular sizes (MW) shown are those of the bands of standard strain 3153A.

of isolates from outpatients or recent admissions and only 20% of isolates from healthy individuals in the community formed clusters. Conversely, the frequency of S_{AB} values of ≥ 0.8 (the threshold defining groups of similarity) was 21% among isolates from patients hospitalized for >3 days, compared with only 7% among isolates from outpatients or recent admissions and 2% among commensal isolates from healthy individuals (Fig. 3B). The differences in the frequency of high S_{AB} values between isolates from hospitalized patients and isolates either from outpatients or recent admissions or from healthy individuals were statistically significant ($P < 0.0010$ and $P < 0.0005$, respectively, by the z test).

We next sought to identify the group(s) of genetically similar strains that was being spread within the hospital. Because of their transmission in the hospital, these strains should occur more frequently in hospitalized patients than in individuals in the community or recent admissions. Figure 4 shows a comparison of the distributions of the three main groups of similar strains within populations of healthy individuals in the community, outpatients or recent admissions, and hospitalized patients. Members of one of the clusters, WB, were isolated with approximately the same frequency (15 to 17%) both outside and within the hospital environment. Members of the cluster WC were in contrast isolated from only 7% of individuals belonging to the group of outpatients or recent admissions plus healthy subjects but from 38% of hospitalized patients, and this difference was statistically significant (z test, $P < 0.005$). The differences in the frequency of isolation of strains from cluster WC between hospitalized patients and either outpatients or recent admissions (38 versus 6%) or healthy individuals in the community (38 versus 10%) were also statistically significant (z test, $P < 0.010$ and $P < 0.050$, respectively). The presence of strains of this cluster was not restricted to a limited number of wards. The 12 hospitalized (>3 days) patients from whom WC strains were isolated had been admitted to 10 different wards.

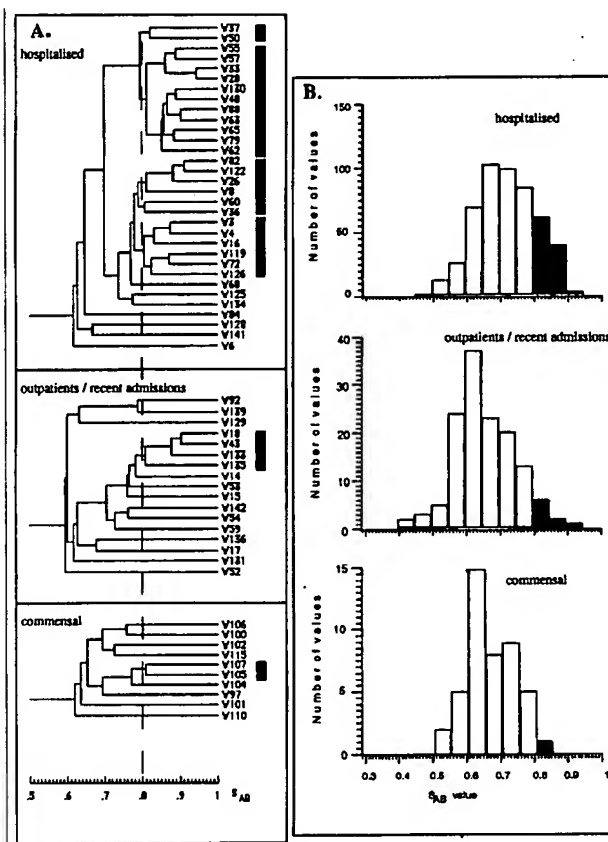


FIG. 3. Comparison of genetic diversity among groups of Wellington isolates from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals in the community (commensal). (A) Dendrograms of each of the three groups. The dashed lines mark the threshold defining groups of genetic similarity ($S_{AB} = 0.8$); the groups are marked by bars on the right of the dendrogram. (B) Histograms of the distribution of S_{AB} values among isolates from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals from the community. Shaded bars mark S_{AB} values of >0.8 .

WC thus represents a widespread hospital-specific group. Strains belonging to the cluster WA were somewhat more frequently isolated from hospitalized patients (frequency of isolation = 19%) than from individuals outside the hospital (frequency of isolation = 8%), but this difference was not statistically significant. When individual wards were analyzed, it became apparent that strains of this group were found more often on patients from each of two of the wards than on individuals from the remainder of the sample. WA strains were isolated from 3 of 7 (or 43%) ward G patients but from only 6 of 60 (or 10%) other individuals. WA strains were also isolated from 2 of 4 (or 50%) ward H patients but from only 7 of 63 (or 11%) other individuals. The number of patients in the two wards was small, but the differences in frequency were statistically significant (z test, $P < 0.050$ and $P < 0.025$, respectively). All of the ward G and ward H patients from whom the WA strains were isolated had been hospitalized for more than 3 days, with one exception: patient W132 had been admitted only 1 day before sampling but had been previously admitted to the same ward only 2 weeks earlier. WA strains may thus also be a group that is being transmitted within the hospital, though only within a limited number of wards.

We next sought to determine if isolates in any of the clusters WA, WB, and WC were more frequently isolated from any

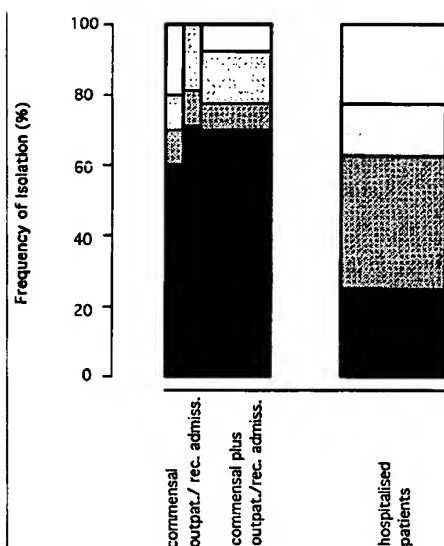


FIG. 4. Frequency of isolation of the major groups of genetically similar Wellington strains, WA (□), WB (▤), and WC (■), from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals in the community. ■, other strains.

subgroups of patients defined by age, gender, predisposing conditions, or body site of isolation, indicating adaptation of groups of genetically similar strains to patients with certain physiological or clinical features. We also tested whether any of the clusters were more frequently isolated from patients for whom clinical features of candidiasis had been reported. No statistically significant correlations were observed.

Analysis of Auckland isolates. Sixty-one *C. albicans* isolates, each from a different individual, were analyzed (Fig. 5). These included 10 commensal isolates (labelled C in Fig. 5) and 51 patient isolates. Of the 51 patients, 21 had been hospitalized for more than 3 days prior to the isolation of *C. albicans* (labelled H in Fig. 5). Twenty-six either were outpatients or had been hospitalized for 3 days or less (labelled OR in Fig. 5). Four additional patients were recent admissions but had been hospitalized within the 6 preceding months and were for that reason not included in the outpatient-or-recent-admission category.

Figure 5 gives an overview of the genetic relationships among the 61 strains. As in Wellington, many of the strains were genetically dissimilar but several groups of genetic similarity were also apparent. The two largest, containing 13 and 11 isolates, respectively, are labelled AA and AB in Fig. 5. Examples of the actual Ca3 patterns are shown in Fig. 6.

In contrast to the one resulting from our analysis of the Wellington isolates, the dendrogram in Fig. 5 does not suggest that isolates from hospitalized patients in Auckland were frequently derived from the main clusters of genetically similar strains. This impression was confirmed by the results of further tests which gave no indication of transmission in Auckland Hospital: an analysis of the genetic diversity of isolates from hospitalized patients, outpatients or recent admissions, and healthy individuals (Fig. 7) showed that isolates from hospitalized patients were not less diverse than isolates from outside the hospital environment were. In the dendrograms in Fig. 7A, 42% of the isolates from hospitalized patients formed clusters with other similar isolates compared with 69% of isolates from outpatients or recent admissions and 30% of commensal isolates. Conversely, the frequency of high S_{AB} values (≥ 0.8) was 9.5% among isolates from hospitalized patients, compared

with 18% among isolates from outpatients or recent admissions and 7% among isolates from healthy individuals in the community. There was also no significant difference in the frequency with which strains from the major clusters, AA and AB, were encountered within and outside the hospital environment (Fig. 8). Likewise, no association of either group of strains with patients on any specific ward(s) was found.

Hospital-specific transmission could have been masked in the above-described analysis by strong preferences of groups of genetically similar strains for particular types of patients (defined, for instance, by physiological or clinical condition) or body sites, regardless of hospitalization. We therefore tested whether either of the two large groups of genetically similar strains was more frequently encountered in subgroups of patients defined by age, gender, predisposing conditions, reported clinical symptoms of candidiasis, or body site than on the remainder of the patients. We found the following statistically significant (z test, $P < 0.05$ or less) correlations. Strains of group AA were encountered 3.4 times more often in patients at high risk of acquiring candidiasis (human immunodeficiency virus (HIV)-positive and cancer patients), and 2.9 times more often in the urinary tract than at other sites. Strains of group AB were encountered 6.4 times more often in patients above the age of 50 than in other patients. None of these prevalences were higher for hospitalized patients than for outpatients or recent admissions, which would have indicated transmission in the hospital between certain patient groups.

The prevalence of group AA strains in the urinary tract was actually highest outside the hospital environment, and the high-risk patients were almost exclusively outpatients or recent admissions. These two factors could thus have concealed a reduced genetic diversity among isolates from hospitalized patients. We therefore repeated the comparison of genetic diversity between isolates from hospitalized and nonhospitalized patients with a sample of isolates from which urinary tract isolates and isolates from high-risk patients had been omitted. The results (data not shown) provided neither evidence nor even a trend pointing towards either reduced genetic diversity among isolates from hospitalized patients or an increased frequency of the remaining cluster AA and cluster AB isolates in hospitalized patients—again providing no indication of hospital-associated transmission.

Whereas the above-described analyses yielded no indication of transmission in Auckland Hospital, they did highlight some differences between the strains isolated from patients and those isolated from healthy individuals. The level of genetic diversity of strains from outpatients or recent admissions was significantly lower (z test, $P < 0.025$) than was that of strains from healthy individuals (18 versus 7% of S_{AB} values of ≥ 0.8). In addition, strains from groups AA and AB were isolated from 14 and 24% of hospitalized (>3 days) patients, respectively, and from 34 and 23% of outpatients or recent admissions but were not isolated from any of the 10 healthy individuals. With the exception of the difference in frequency of isolation of AA strains between hospitalized patients and healthy individuals, all these differences were statistically significant (z test, $P < 0.050$ or less). The populations of strains found in both outpatients or recent admissions and hospitalized patients were thus not identical to the population of commensal strains in healthy individuals.

DISCUSSION

It was the aim of this study to assess whether *C. albicans* strains can be transmitted to patients within the hospital environment by using a retrospective comparison of isolates ob-

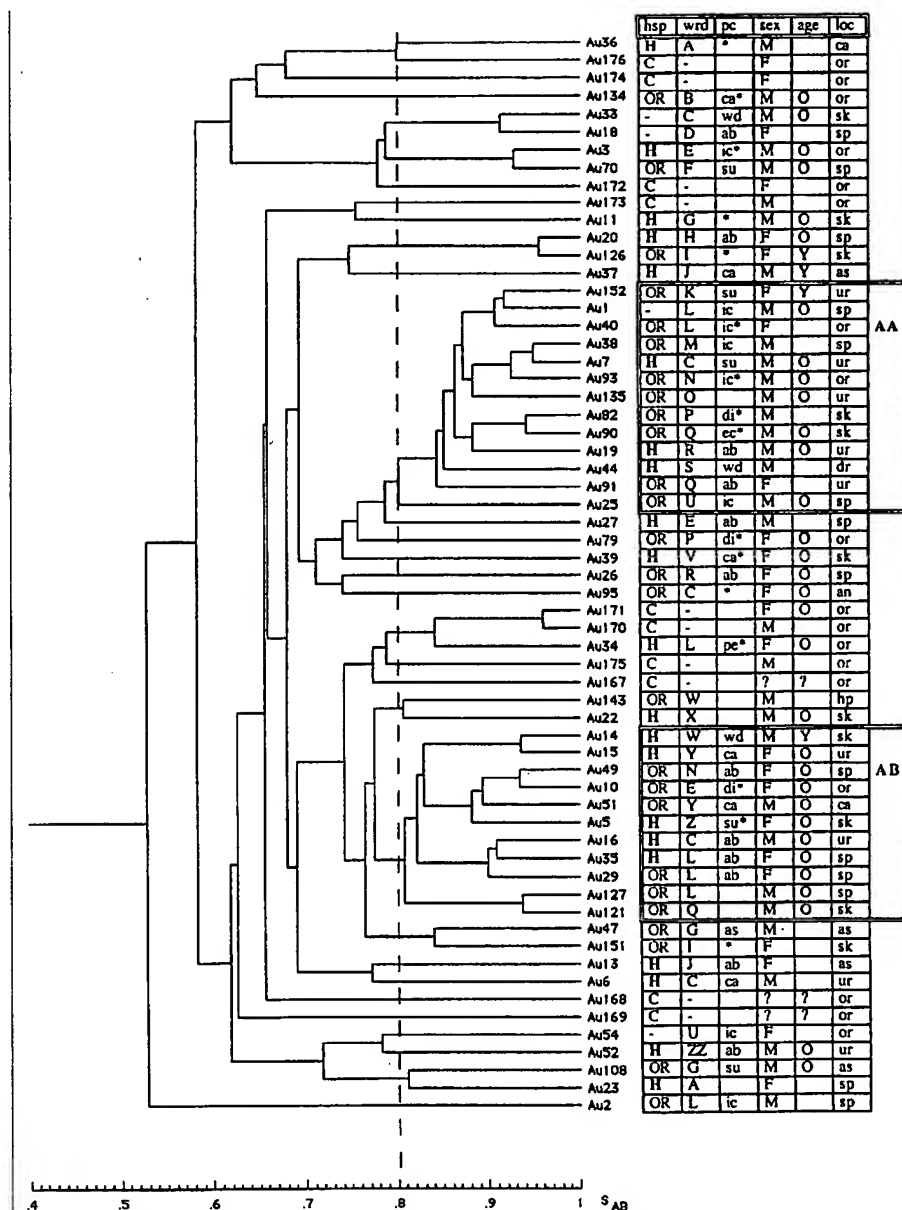


FIG. 5. Genetic relationships among all Auckland isolates determined on the basis of the similarity of their Ca3 fingerprints. The dashed line in the dendrogram denotes the threshold S_{AB} value of 0.8 for defining groups of genetic similarity. The two largest groups of genetically similar isolates, AA and AB, are marked on the right of the dendrogram. Following the name of each isolate is information on the duration of hospitalization of the patient (column labelled hsp; H, hospitalized for >3 days; OR, outpatient or recent admission; -, recent admission who was hospitalized previously; C, individual in the community (commensal isolate)), the wards to which patients had been admitted (column labelled wrd), the clinical status indicating conditions predisposing the patient to candidiasis (column labelled pc; ab, antibiotics; as, asthma; ca, catheterization; di, diabetes; ec, eczema; ic, immunocompromised by HIV, leukemia, or cancer or cancer treatments; pe, pleural effusion; su, surgery; wd, wound). Asterisks denote patients for whom clinical symptoms indicating candidiasis were reported, sex, age (O, >50 years; Y, <2 years; blank cells, 2 to 50 years), and body site or material from which the isolate was obtained (column labelled loc; an, anus; as, aspirate; ca, catheter; dr, drain; hp, hip; or, oral cavity; sk, skin, including wounds; sp, sputum; ur, urine).

tained from patients who had been hospitalized for different lengths of time. In one of two hospitals surveyed (Wellington Hospital), the majority of isolates obtained from patients after prolonged hospitalization was derived from a limited number of groups of genetically similar strains. Strains from outpatients or recent admissions and healthy individuals were, in contrast, significantly more dissimilar to each other, i.e., genetically more diverse. We identified two groups of genetically

similar strains which were significantly more prevalent in patients after prolonged hospitalization, one (WC) found in patients from a large number of different wards and one (WA) found in patients from two specific wards. No other patient characteristics, such as sex, age, or physiological or clinical state, were correlated with the increased prevalence of these clusters. These observations thus indicate that in Wellington Hospital, hospitalization led to the frequent replacement of

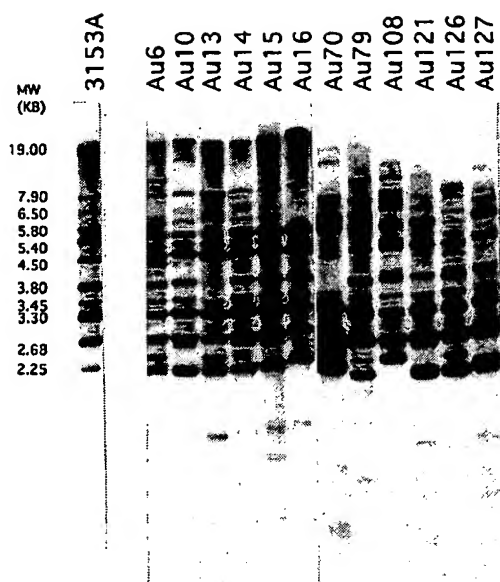


FIG. 6. Range of DNA fingerprints of isolates from different individuals in Auckland plus strain 3153A, which is included as a standard. Molecular sizes (MW) shown are those of the bands of standard strain 3153A.

patients' strains with strains acquired in the hospital environment and therefore provide evidence (albeit indirectly) for nosocomial transmission of *C. albicans*.

The fact that our evidence indicates transmission of groups of similar strains rather than of a single strain allows some conclusions to be drawn regarding the possible scenario underlying these transmissions. It seems likely that the progenitors of the two groups of strains transmitted became established in the hospital in the past and that their progeny have since evolved while spreading throughout the hospital environment; such spread may involve not only contact between human hosts but also inanimate surfaces and food (21). We are now in the process of identifying the reservoirs of these strains and vehicles of their transmission in the hospital environment. Our findings also suggest one reason why previous attempts to demonstrate nosocomial transmission of *C. albicans* have yielded inconclusive results (5). Since the methods employed lacked the capability to group strains according to their similarity, they could have detected only single-strain transmission and not transmission of groups of genetically similar strains.

In the second hospital studied (Auckland Hospital), we found no indications of nosocomial transmission of *C. albicans*. We emphasize that this does not imply that compromised patients in this hospital always retained their commensal strains. The frequency of the major groups of strains, AA and AB, in patients (both hospitalized patients and outpatients or recent admissions) was higher than their frequency in healthy individuals. In addition, the level of genetic diversity of patient isolates was lower than that of commensal isolates. These differences indicate that commensal strains had been replaced but that replacement had occurred regardless of hospitalization, implicating the community as the source of the replacing strains. This assumption is corroborated by the results of an earlier study in which we demonstrated strain replacement in nonhospitalized AIDS patients (13). The design of the present study did not allow us to determine when strain replacement in hospitalized patients took place. On the basis of our earlier work (13), it seems likely that replacement may have occurred

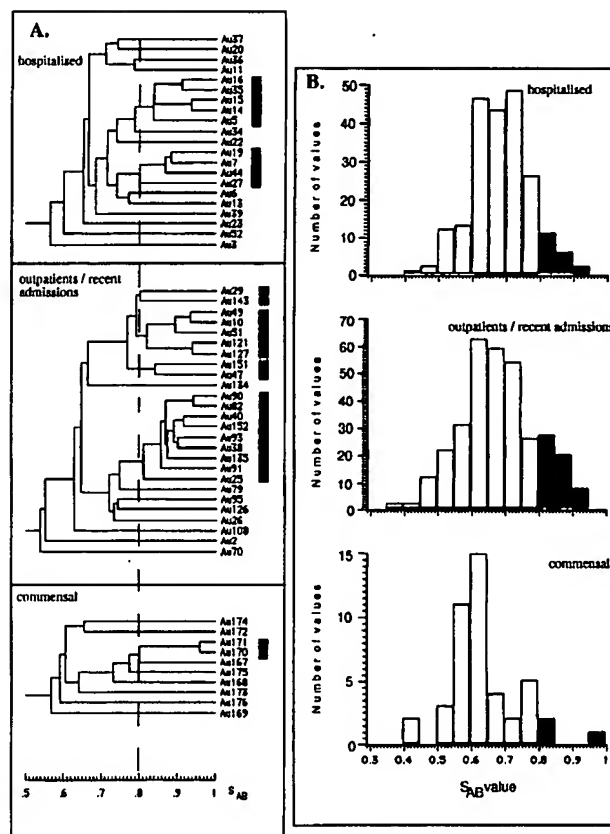


FIG. 7. Comparison of genetic diversity among groups of Auckland isolates from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals in the community (commensal). (A) Dendrograms for each of the three groups. The dashed lines mark the threshold defining groups of genetic similarity ($S_{AB} = 0.8$); the groups are marked by bars on the right of the dendrograms. (B) Histograms of the distribution of S_{AB} values among isolates from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals from the community. Shaded bars mark S_{AB} values of >0.8.

prior to the patients' hospitalization. Certain groups of replacing strains were found more frequently on patients with particular characteristics, regardless of whether the patients were hospitalized: group AA preferably associated with high-risk patients and patients with urinary tract disorders, and group AB preferably associated with patients older than 50 years. This, in turn, raises the possibility of specific adaptation of groups of strains towards colonization of certain types of patients. However, in a combined analysis of isolates from a number of New Zealand hospitals these strains grouped together with strains from other centers showing no such preferences (12). This makes it more likely that the preferred association of the Auckland groups with certain types of patients was based on routes of transmission existing within these patient populations.

The observation that nosocomial transmission of *C. albicans* apparently occurs only in some hospitals and not in others raises questions as to the reasons for these differences. One possible explanation would be that the groups of strains transmitted in Wellington Hospital were genetically well adapted to survival in the hospital environment and that strains with such capabilities were not present in Auckland. This appears unlikely because in a combined Ca3 analysis of isolates from several hospitals in New Zealand (12), we found that the

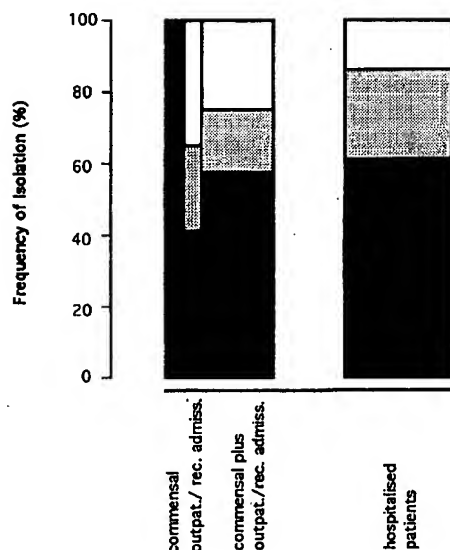


FIG. 8. Frequency of isolation of the major groups of genetically similar Auckland strains, AA (□) and AB (▨), from hospitalized (>3 days) patients, outpatients or recent admissions, and healthy individuals in the community (commensal). ■, other strains.

strains transmitted in Wellington (groups WA and WC) formed clusters of genetic similarity with Auckland strains belonging to groups AA and AB. Further studies will be required to assess which epidemiological factors may be responsible for the observed differences in the frequency of transmission between the two hospitals.

In addition to providing evidence for nosocomial *C. albicans* transmission, the results of this study suggest potential applications for Ca3 fingerprinting in preventing candidiasis outbreaks. Because of the high resolution of Ca3 fingerprinting, occasional spot testing of fairly small numbers of select isolates submitted to the clinical laboratory is apparently sufficient to determine whether transmission of *C. albicans* occurs in a given hospital, even in the absence of an obvious outbreak situation. Ca3 fingerprinting is thus a sensitive first screen for the detection of nosocomial transmission of *C. albicans*, and if the fingerprinting analyses were contracted out to laboratories in which the methodology is established, such screens would also be rapid and inexpensive. In instances in which such a spot test indicated transmission, the method could potentially be utilized to track reservoirs and routes of transmission by sampling of the hospital environment and caregivers. This may enable a hospital to eliminate such routes before they become the means of transmission of highly virulent strains causing single-strain outbreaks.

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MANUAL OF CLINICAL MICROBIOLOGY

Third Edition

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**AMERICAN SOCIETY
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Section V. Laboratory Tests in Chemotherapy

Chapter 41

General Considerations

JOHN C. SHERRIS AND JOHN A. WASHINGTON II

A number of considerations are involved in selecting an appropriate antimicrobial to treat an infection. (The term antimicrobial is used in this section to describe both antibiotics and chemotherapeutics.) These include: (i) knowledge of the inherent *in vitro* susceptibility of the infecting organism to appropriate antimicrobics; (ii) the relationship of the susceptibility of the strain to that of other members of the same species; (iii) pharmacological properties including toxicity, protein binding, distribution, absorption, and excretion, particularly under circumstances of existing or developing hepatic or renal failure; (iv) previous clinical experience of efficacy in treating infections due to the same species; (v) the nature of the underlying pathological process, its natural history, and its influence on chemotherapy; and (vi) the immune status of the host.

Of these factors, the concentrations of antimicrobial required to inhibit or kill organisms *in vitro* and those attained in body fluids during treatment are subject to direct measurement in the clinical laboratory. The purpose of this section of the manual is to provide detailed descriptions of appropriate procedures for these purposes. The susceptibility methods described are for use with bacteria other than mycobacteria, which are separately considered in Chapter 14.

The role of the laboratory in the selection and monitoring of chemotherapy was succinctly expressed by Theodore G. Anderson in the first edition of this manual: "When selecting an antimicrobial agent for therapy, it is the physician's responsibility to take into consideration the pharmacological characteristics of the several drugs as well as their relative antimicrobial effectiveness. The responsibility of the laboratory is to provide information through standardized *in vitro* tests, of the activity of appropriate antimicrobial agents against the organism in question." The methods given in the subsequent chapters constitute accepted approaches among the authors providing this information. Different procedures have been developed by others in a

number of countries, and the reader is referred to more detailed reviews for further information and for broader consideration of the theory of the subject (1, 2, 4, 5, 8, 10, 13-17).

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Influence of technical variation on susceptibility test results

The results of both dilution and diffusion susceptibility tests may be influenced markedly by the reagents and conditions of the tests, and these variables have been the source of considerable confusion in the past. Inoculum density is especially important. In addition, incubation time and temperature, pH, atmosphere, and stability of antimicrobics may all influence the endpoints obtained. Differences in constituents or ionic content of the medium, even between batches, may influence results of tests, particularly with the sulfonamides, tetracyclines, polymyxins, and aminoglycosides. In addition, diffusion tests are influenced by the growth rate of the organism and by the type, depth, and concentration of the agar used. For these reasons, special emphasis has been placed on reference procedures and methodological standardization (4, 6, 7, 11, 12, 19, 20), because only in this way can adequate reproducibility be obtained in investigative and clinical work.

In each of the susceptibility tests described, the inoculum is derived from several colonies. This is designed to reduce the chance of selecting variants derived from loss-mutations (e.g., loss of penicillinase production in staphylococci) or segregants from R-factor resistance markers. It also increases the chance of including representatives of the more resistant organism if more than one strain is represented by colonies that cannot be distinguished morphologically. The final inocula are reasonably heavy, which increases the chance of detecting high-frequency mutations to resistance and heteroresistant strains. The media selected show generally good

buffering qualities and reproducibility and are of physiological pH. A central criterion of the conditions to be used in effective diffusion, dilution, or automated tests is that they must be able to detect strains carrying clinically important resistance determinants.

Selection of susceptibility tests methods

Diffusion test. The most widely used procedure is still the disk diffusion method, described in Chapter 44, which has been accepted by the Food and Drug Administration (FDA) (6, 7) and as a standard by the National Committee on Clinical Laboratory Standards (NCCLS) (11). This procedure, as normally used, is essentially a qualitative test which allocates organisms to the sensitive (susceptible), intermediate (or indeterminate), or resistant categories discussed below. The procedure is flexible in regard to the antimicrobics that can be tested, and it is easy to set up individual tests at different times. It is technically simple, although it requires careful attention to detail. It is generally applicable to organisms whose growth rate approximates those of the members of the *Enterobacteriaceae* family and *Staphylococcus aureus*, and the procedure has now also been adapted to detect penicillinase-producing strains of *Haemophilus influenzae* and *Neisseria gonorrhoeae*, and strains of pneumococci that have developed increased resistance to penicillin and some other antibiotics (see Chapter 44). In cases of clinical urgency, clinical material may serve as the inoculum for the test if the precautions indicated in Chapter 44 are followed. More experience has been gained over the years with this diffusion procedure than with any other test.

The deficiencies of the diffusion test are its nonquantitative interpretation, its inapplicability to many slow-growing organisms and anaerobes, and its inaccuracy in predicting susceptibility (as opposed to resistance) with antimicrobics, exemplified by the polymyxins, that diffuse poorly. Overall, however, it is an effective procedure for most routine tests but requires supplementation with a dilution test in situations when it is inapplicable or when more quantitative results are needed.

Dilution test. The most quantitative method for antimicrobial susceptibility testing is one of the dilution tests (considered in Chapters 42, 43, and 47) which are derived from the International Collaborative Study recommendations (4) or from proposed NCCLS standards. These yield direct quantitative results, are essentially uninfluenced by the growth rate of the organism, and avoid some of the complexities of diffusion properties of antimicrobics. Dilution tests do not

have the flexibility of the diffusion test, generally cannot be used for direct tests of clinical material because of the difficulty in detecting contamination, and, if reported quantitatively, require that the clinician be able to interpret the result or be helped in doing so.

The primary indication for dilution tests is to obtain quantitative susceptibility data when these are important or necessary for proper management of antimicrobial therapy. Although qualitative data are usually adequate for guiding the therapy of most infections, quantitative information may be needed when drug dosage schedules and serum levels must be closely monitored or under the conditions in which disk test results are inapplicable, equivocal, or unreliable. These conditions include tests on slow-growing organisms, confirmation of susceptibility (as opposed to resistance) to the polymyxins (B or E), confirmation of resistance to the aminoglycosides (particularly gentamicin, tobramycin, and amikacin), and tests with potentially toxic but clinically useful antimicrobics which yield intermediate susceptible results by the disk test. Infections due to microorganisms which are categorized by the disk test as resistant or intermediate to the relatively nontoxic penicillins and cephalosporins may occasionally be treated preferentially and safely with large doses of one of these agents. Some urinary tract infections may respond to ordinary dosages of some antimicrobics because of the high levels which they attain in the urine. In these cases, the precise degree of susceptibility of an organism may influence the choice of antimicrobial, its dosage, and its route of administration. Other indications for dilution methods are for testing the susceptibilities of anaerobes by the methods described in Chapter 45 and for determining bactericidal activity or evidence of synergism or antagonism between antimicrobics against particular microorganisms. These procedures are considered in Chapter 46. Finally, dilution tests have been found to be practical and economical for routine purposes through the use of semiautomated microdilution techniques (see Chapter 43) or replica-plating agar dilution methods (see Chapter 42).

Any laboratory that intends to use the dilution test routinely and to prepare its own reagents and antibiotic dilutions must have the ability to prepare and maintain fully potent stock solutions of antimicrobial and to produce working dilutions on a regular basis. As with all susceptibility tests, the laboratory must control inoculum size and endpoint reading and must develop or use a quality control system that will give endpoints within the range of each series of antimicrobial dilutions. Recent data from profi-

ciency testing surveys show rather wide variation in quantitative results on the same strain tested in different laboratories by a variety of dilution procedures and schedules. Interlaboratory reproducibility is much better when well-controlled prediluted commercial systems are used, and similar improvement can be expected if the protocols used in Chapters 42 and 43 are followed.

Routine use of dilution procedures that do not use preprepared commercial plates is most appropriate for larger laboratories where there is sufficient skilled technical help to ensure reproducibility. Preprepared systems, when adequately controlled, are applicable to smaller laboratories.

Tests for antimicrobial inactivating enzymes. In some instances, resistant strains of bacteria among originally susceptible species owe their resistance exclusively to their ability to destroy or inactivate particular antibiotics. This is the case with penicillin and ampicillin resistance of strains of *Staphylococcus aureus*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae* that produce penicillinase. These can be detected rapidly and accurately with simple chemical procedures for detection of the enzyme (18), and results are available much more rapidly than those of orthodox susceptibility tests. Procedures for this purpose are described in Chapter 46.

Automated tests. Recently, a variety of mechanized or automated procedures have been developed for susceptibility testing, and these are considered in Chapter 48. Some facilitate the performance and reading of traditional overnight susceptibility tests. Others are designed to yield qualitative or limited quantitative results on the same day that the test is set up. Several of these procedures have already been evaluated by collaborative studies and have been shown to have a high degree of reproducibility. However, comparability of procedures providing rapid results with overnight dilution or diffusion tests has been more difficult to achieve with a few organism-antimicrobial combinations because the extent of inhibition of growth in the first few hours of contact may differ from that seen after overnight incubation. Most of these difficulties are being overcome by the use of heavier inocula or computer analyses of growth patterns in the presence of one or more concentrations of antimicrobial. These approaches are discussed in more detail in Chapter 48. Suffice it to say that automated methods have already been shown to have a place in routine work of larger laboratories when their limitations are recognized and avoided by using a traditional test on organism-antimicrobial combinations for which the automated test is inappropriate.

Interpretation of susceptibility tests: "susceptibility" and "resistance"

The interpretation of a quantitative susceptibility test result has three major components.

1. *The relationship of the MIC (minimal inhibitory concentration) or MLC (minimal lethal concentration) of the organism to the concentration of antimicrobial in the blood, or in some cases urine or other fluid, obtained with the dosage given.* This has proved a clinically useful approach, but is inevitably an incomplete model of the in vivo situation because of the varying degrees of protein binding, the interacting effects of host defense mechanisms, and the arbitrary aspects of the selection of test conditions.

2. *The relationship of the susceptibility of the strain under test to that of other members of the same species.* This is useful because the selection of resistant mutants or strains with extrachromosomal determinants of resistance has led to the appearance of populations of strains of some species well separated from the "wild" types that were previously uniformly susceptible to the antimicrobial. The resulting bimodal distribution of susceptibilities correlates well with clinical responsiveness. Thus, a strain falling in the more resistant population is considered a priori a resistant member of that species.

3. *Clinical experience with the treatment of the particular type of infection involved.* An ideal interpretation of susceptibility test results takes account of these factors independently. From a practical point of view, organisms are frequently allocated to predetermined "susceptible," "resistant," and one or more "intermediate" categories, and this approach was considered by the International Collaborative Study to continue to be useful and necessary in the light of presently available technical methods and general understanding of the principles of chemotherapy (4). The three categories recommended for the diffusion test given in Chapter 44 have been based on the synthesis of the first two criteria given above. They have been defined (11) as: (i) susceptible (or sensitive), implying that an infection due to the strain tested may be appropriately treated with the antimicrobics and dosages recommended for that type of infection and infecting species, unless otherwise contraindicated; (ii) resistant, containing strains not completely inhibited within the usual therapeutic dosage range; and (iii) intermediate, comprising a "buffer zone" which prevents major interpretative discrepancies that might result from small uncontrolled technical factors. The last category also includes strains which may respond to concentrations attainable by unusually high dosage or in areas, such as portions of the urinary tract, where the antimicrobial is concen-

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tions given in the first paragraph of this chapter.

This three-category system requires qualifi-
cation in that it does not consider the blood
levels that may be attained with very high dos-
ages of the relatively nontoxic penicillins and
cephalosporins, the high urine levels of certain
antimicrobials, or the low blood levels achieved
with oral as opposed to parenteral dosage of
certain antimicrobics. Thus, it is important to
recognize the need for quantitative data in spe-
cial cases.

Indications for susceptibility tests in the clinical laboratory

Tests are indicated for organisms contributing
to the infectious process whose susceptibility
cannot be predicted from knowledge of their
identity. This applies, in particular, to *S. aureus*,
to gram-negative enteric organisms, to some an-
aerobes, and to unusual and opportunistic spe-
cies playing a pathogenic role. Specific patterns
of susceptibility (antibiogram) may be charac-
teristic of a species and may, therefore, assist in
correct species allocation. Antibiograms may be
determined for epidemiological reasons because
the occurrence of an unusual antibiogram for a
given species often assists in the recognition of
common-source outbreaks and patterns of cross-
infection.

Routine susceptibility tests are not needed
when resistance has not been described to the
antimicrobial of choice, e.g., *Streptococcus py-
ogenes* and *Neisseria meningitidis* to penicillin.
However, this situation needs to be reviewed
constantly, because the recent emergence of β -
lactamase-producing strains of *H. influenzae*
and *N. gonorrhoeae* (3) and of pneumococci
resistant to penicillin and other antibiotics (9)
has changed the requirements for testing among
these species. Susceptibility testing should be
avoided on members of the normal flora in their
normal habitat and on organisms that are known
not to be playing a pathogenic role. To make
such tests is both wasteful and misleading. The
routine diffusion susceptibility test described in
Chapter 44 should never be made on organisms
for which its interpretative criteria are inap-
plicable (e.g., slow growers or anaerobes).

Selection of antimicrobics for testing

For routine susceptibility testing, single rep-
resentatives of a group of related antimicrobics
are used whenever possible, and the FDA has
recognized specific class disks for many anti-
microbic groups for use in the diffusion test (see
Chapter 44). However, there are sufficient dif-
ferences in the spectra of activity, or mecha-

nisms of resistance, among certain penicillins,
cephalosporins, and aminoglycosides that more
than one representative member of each of these
groups needs to be tested. The selection of an-
timicrobics to be tested should also be limited
to those that are clinically useful and appropri-
ate for the site of infection, except when the
procedure is being used to determine antibio-
grams for epidemiological purposes or when par-
ticular antimicrobics yield taxonomically useful
information. For example, tests with nitrofur-
antoin and nalidixic acid should be limited to
bacteria isolated from the urinary tract, and
tests of the combination of trimethoprim and
sulfamethoxazole should be limited to organisms
causing urinary tract infections or otitis media,
which are presently the only approved uses of
this combination in the United States. Tests
with methenamine mandelate should not be per-
formed because its *in vivo* activity depends on
urinary acidification to a pH of 5.0 or less, and
this condition is not reproduced in the ordinary
test systems.

The antimicrobics listed in Table 1 should
fulfill the basic requirements for routine tests in
most clinical laboratories, for aerobic and fac-
ultatively anaerobic bacteria, and the list conforms
closely to NCCLS recommendations. Additional
antimicrobics may be added as noted in the
footnotes to Table 1, when needed for special
problems of the individual patient, or to take
account of local preference or rules. Antimicro-
bics appropriate for testing anaerobes are con-
sidered in Chapter 45. To avoid confusion, re-
sults and tests on antimicrobics that are inap-
propriate in therapy and that are tested for
epidemiological or taxonomic purposes should
not be reported to the physician.

A decision to include a new antimicrobial in
the basic test set of a routine laboratory should
be based on (i) the use of the antimicrobial having
been approved by the FDA or its equivalent, (ii)
an activity spectrum differing significantly from
presently used analogs, (iii) readily available
information on its clinical pharmacology, (iv)
the availability of well-documented and FDA-
approved zone diameter interpretative criteria
in the case of disks for diffusion testing, and (v)
a clear clinical need for its introduction.

SPECIAL TESTS AND ASSAYS

Susceptibility tests make up the bulk of the
clinical laboratory tests which are ordered to
assist the clinician in his choice of chemothera-
peutics. They may need to be supplemented
with other procedures in certain complex clinical
situations, especially in subacute bacterial en-
docarditis and in severe infections in the immu-
nologically compromised. In these cases, deter-

TABLE 1.
Basic sets of antimicrobics to be tested routinely against rapidly growing aerobic and facultatively anaerobic bacteria^a

Antimicrobics	Staphylococci and streptococci	Enterococci	Enteric gram-negative bacilli other than <i>P. aeruginosa</i>		<i>P. aeruginosa</i>
			Urinary	Other	
Penicillins					
Ampicillin		1	1	1	
Carbenicillin			1	1	1
Nafcillin, oxacillin, or methicillin ^b	1				
Penicillin G	1	1			
Cephalosporins					
Cefamandole	2		1 ^c	1 ^c	
Cefoxitin	2		1 ^c	1 ^c	
Cephalothin	1		1	1	
Chloramphenicol	2	2		2	
Clindamycin	1				
Erythromycin	1	1			
Aminoglycosides					
Amikacin			1	1	1
Gentamicin	2		1	1	1
Kanamycin	2		1	1	
Tobramycin			1	1	1
Polymyxin B or E			2	2	1
Tetracycline	2	2	1	1	
Vancomycin	2				
Urinary tract agents					
Nalidixic acid			1		
Nitrofurantoin			1		
Sulfonamides			1		
Sulfamethoxazole/tri-methoprim			1		

^a 1 = Primary set; 2 = secondary drugs.

^b Oxacillin or nafcillin is preferable for detecting heteroresistant methicillin-resistant *S. aureus*.

^c Cefamandole and cefoxitin may be reserved for testing cephalothin-resistant organisms only.

mination of bactericidal concentrations or of the effect of combinations of antimicrobics may need to be measured. Direct tests of the ability of the antimicrobial in the patient's serum to inhibit or kill the infecting organism may also be helpful in monitoring the adequacy of dosage schedules (see Chapter 46). So far, there is still no general agreement on methods for determining minimal lethal (bactericidal) concentrations, studying antimicrobial combinations, or determining serum inhibitory or bactericidal activity against the infecting organisms. The procedures given should continue to serve as a basis for further studies towards methodological standardization of these important tests.

Increasingly, it is necessary to determine the amount of antimicrobial present in serum, urine, other fluids, or tissues. In clinical practice, this applies particularly to agents such as gentamicin, tobramycin, and amikacin, for which potentially toxic levels and therapeutic levels are very close. Serum assays are thus required to ensure that antimicrobial concentrations in the blood

are within a safe, but effective, range. This is particularly the case in patients with renal deficit, in whom serum levels of antimicrobial may be less predictable. Simple, rapid, and accurate methods for this procedure are given in Chapter 47.

FUTURE NEEDS

Since the last edition of this manual, an NCCLS standard for diffusion susceptibility tests has been published and an updated revision has recently appeared (11). A standard for anaerobic susceptibility testing (12) has also been published, and one for dilution tests is in the final stages of preparation. These provide reference points for clinical, epidemiological, and research studies. Concurrently, quality control values for standard strains have been developed and updated to include new antimicrobics. Major proficiency testing programs for susceptibility test results are now operating through the Center for Disease Control and the College of American Pathologists. Thus, the background

for improvement of susceptibility test procedures to give greater accuracy and better inter-laboratory reproducibility has been laid. It is especially important that mechanisms be maintained for regular updating of reference procedures, interpretative standards for new antimicrobics, recommendations for basic routine sets of antimicrobics for testing, and data on performance of quality control strains. Supplements providing such data are planned by the NCCLS.

Developments in mechanization and commercially available test kits have now brought the capacity for quantitative susceptibility testing even to small laboratories, and this trend is likely to continue. Automated procedures that yield rapid results and can be directly interfaced with computer reporting systems are already available, and more can be expected to be developed. There is a clear need for agreement on evaluative protocols by which the relationship between the performance of new systems and reference procedures can be assessed and for definitions of acceptable performance. Potential purchasers need access to such data before deciding whether to incorporate a system into their routine work.

Media used for susceptibility testing remain a problem. Performance standards for commercially produced Mueller-Hinton media are needed for application at the manufacturer's level. For instance, it has become increasingly apparent that there are variations in performance of *Pseudomonas aeruginosa* with the newer aminoglycoside antimicrobics when tested with different methods and with batches of media from the same or different manufacturers, resulting in MICs ranging over several dilution steps. This has caused problems of interpretation that can only be resolved by performance control at the manufacturer's level. With the wider acceptance of reference procedures, there is need for a review of qualitative interpretative categories used in diffusion and in some dilution and automated procedures. The boundaries of interpretative categories have involved some best-judgement types of decisions and subjective clinical experience. Moreover, validation of categories by detailed clinical studies has been made difficult in the past by inadequate methodological standardization. This should now be possible, particularly with new antimicrobics.

Methodological agreement is needed on techniques for determining bactericidal endpoints, for measurements of the effects of combinations, and for performing serum inhibitory or bactericidal tests. In the absence of standardized methods or reference procedures, results from different laboratories cannot be compared with con-

fidence, and an adequate base of experience has not been developed for fully satisfactory interpretation of the results. For all of these procedures, the kinetics of microbial killing by antimicrobics make it essential that statistical endpoints be accepted, such as those recommended in Chapter 46.

In summary, we may reasonably look forward to improved performance of media and of procedures for orthodox susceptibility tests, to improvements in selecting and disseminating interpretative recommendations, and to improved quality control. Beyond that, rapid automated or semiautomated procedures for susceptibility testing are coming into use and should result in better reproducibility through elimination of many sources of technical error. There continues to be a need for reference or agreed procedures for determining lethal endpoints, interactions of combinations, and bactericidal activities of serum so that interpretations of the results of these tests can be refined through cumulative experience. These developments should increase the usefulness of laboratory procedures in the selection and monitoring of chemotherapy.

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Persistence, Replacement, and Microevolution of *Cryptococcus neoformans* Strains in Recurrent Meningitis in AIDS Patients

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Six separate human immunodeficiency virus-positive patients with cryptococcal meningitis were each found to have been infected with a unique strain of *Cryptococcus neoformans* on the basis of genomic DNA fingerprinting analysis with the microsatellite sequence-containing oligonucleotide probe (GGAT)₄ and by random amplification of polymorphic DNA. Two patients (A and B) experienced a recurrent episode of infection. Between 12 and 16 single-colony isolates recovered from primary isolation media (>50% of *C. neoformans* colonies recovered) from cerebrospinal fluid specimens were fingerprinted from both patients during each episode. The fingerprints of both isolate collections from patient B were very similar, although minor polymorphisms were evident in both sets of profiles. The fingerprints of the isolate collection from the initial episode of infection in patient A were also identical to each other, apart from minor polymorphisms, but they were clearly different from the corresponding profiles of the isolate collection from the recurrent episode, the latter of which were completely identical, apart from minor polymorphisms in a single isolate. Furthermore, prolonged storage and in vitro subculture of the isolates did not alter the fingerprint profiles. These results provided convincing evidence that patients A and B were each infected with a single *C. neoformans* strain during each episode of infection and that in patient B, the same strain persisted and caused both episodes, while in patient A, a different strain was responsible for each episode. The prevalence of polymorphisms in multiple single-colony isolates from both patients also suggested that *C. neoformans* populations may undergo microevolution.

Cryptococcus neoformans is an encapsulated basidiomycetous yeast species which occurs naturally in the environment and is frequently associated with pigeon droppings and soil contaminated with avian guano (11). Under most circumstances, inhalation of this organism fails to cause symptomatic infection (14). However, in 5 to 10% of individuals with AIDS, severe life-threatening disease in the form of meningoencephalitis can occur (6). The vast majority of the isolates responsible for these infections are *C. neoformans* var. *neoformans* serotype A (10).

Because of the perceived importance of these organisms as human pathogens, the species has been subjected to intense study during the last decade. In this regard, the recent development and application of techniques designed to differentiate between individual isolates are of particular relevance because of the high incidence of recurrent cryptococcal infections in AIDS patients once antifungal therapy has ceased (20). In order to design effective antifungal drug treatment regimens, it is important to determine if recurrent disease is the result of reinfection with the original strain or infection with a novel strain. Unfortunately, individual strains of *C. neoformans* are morphologically and physiologically indistinguishable, and isolates of *C. neoformans* var. *neoformans* can only be divided into three serotypes (serotypes A, D, and A-D) (9). Consequently, techniques used in the epidemiological analysis of these organisms have concentrated on detecting genetic differences between individual isolates. To date, a wide variety of molecular typing systems have been applied to *C. neoformans* epidemiol-

ogy, including electrophoretic karyotyping (17, 19), PCR fingerprinting (13), random amplified polymorphic DNA (RAPD) analysis (2, 8), multilocus enzyme typing (1, 2), allelic variation of the *URA5* locus (3, 5), and DNA fingerprinting with (i) genomic DNA probes (5, 18, 22, 23, 26, 27), (ii) mitochondrial DNA probes (25), and (iii) oligonucleotide probes homologous to microsatellite sequences (8). The general consensus from many of these studies is that *C. neoformans* strains exhibit considerable genetic heterogeneity and that recurrent infections are apparently due to the persistence of the original infecting strain (17, 22, 23). However, a recent study by Haynes et al. (8) indicated, on the basis of oligonucleotide and RAPD fingerprint analysis of genomic DNA, that in two patients (from five examined in total), recurrent infections may have been due to reinfection with a novel strain. In addition, this study indicated that one patient was coinfecting with more than one strain during a single episode of infection. These data have aroused some controversy (4), and their clinical implications are of sufficient importance to warrant the analysis of additional cases of recurrent *C. neoformans* infection in order to confirm unequivocally that reinfection with novel strains may occur. The purpose of this study was to corroborate these earlier findings by analyzing multiple single-colony isolates recovered from patients during two recurrent episodes of meningitis and to determine the extent of genetic diversity within phenotypically homogeneous populations of *C. neoformans* recovered from the same clinical specimen.

MATERIALS AND METHODS

C. neoformans isolates. *C. neoformans* isolates were recovered from six human immunodeficiency virus-infected individuals with cryptococcal meningitis at The Chelsea and Westminster Hospital, London, United Kingdom, between January 1994 and April 1995. In the case of four of these patients, *C. neoformans* isolates were recovered from a single episode of infection. The remaining two patients,

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both of whom were homosexual males with AIDS, experienced two successive episodes of meningitis. These two patients, termed A and B, respectively, presented with the classical symptoms of cryptococcal meningitis for the first time in November 1994, and in both cases, *C. neoformans* was isolated by plating of aliquots of cerebrospinal fluid (CSF) on Sabouraud's agar. After treatment with 0.6 to 0.7 mg of amphotericin B $\text{kg}^{-1} \text{day}^{-1}$ for 2 to 4 weeks, both patients made a complete clinical recovery. Thereafter, each patient received 200 to 400 mg of fluconazole day^{-1} on an ongoing basis. However, clinical symptoms of meningitis reappeared in patient A in April 1995 and in patient B in March 1995, and in both cases, *C. neoformans* was again isolated from CSF samples. The time intervals between the initial and recurrent isolation of *C. neoformans* from patients A and B were 158 and 112 days, respectively. No additional specimens were taken from either patient during the symptom-free period between episodes of meningitis. Isolates were recovered on Sabouraud's agar after incubation at 37°C for 48 h and subcultured on fresh media prior to identification with the API ID 32C Yeast Identification System (bioMérieux, Marcy l'Etoile, France) and urea assimilation. Each isolate was stored in Protect cryo-storage vials (STC, Heywood, Lancashire, United Kingdom) at -20°C prior to detailed analysis. The CSF specimens from the initial and recurrent episodes of meningitis for both patients A and B each yielded between 10 and 30 *C. neoformans* CFU on primary isolation media. In the case of both episodes of infection in patient A and in the recurrent episode of infection in patient B, 20 individual well-separated *C. neoformans* colonies from the primary isolation plates were selected at random and stored; 16 of these from each isolate collection were subjected to further detailed analysis. In the case of the initial episode of infection in patient B, only 12 *C. neoformans* CFU were recovered on primary isolation media, all of which were stored and subjected to detailed analysis. The isolate collections from the initial episodes of infection in patients A and B were labelled A₁1-20 and B₁1-12, respectively, while those from the recurrent episodes were labelled A₁₁1-20 and B₁₁1-20, respectively.

Fluconazole susceptibility testing. The susceptibility of *C. neoformans* clinical isolates to fluconazole was determined by broth microdilution in RPMI 1640 medium (15). Isolates were grown in 96-well microtiter plates (Corning) incubated at 35°C for 72 h with agitation (16). An end point of 80% growth inhibition (IC₈₀) was determined for each isolate by measuring the A₄₀₅ with an automated microplate reader (Spectra I; SLT-Lab Instruments, Salzburg, Austria).

DNA fingerprinting. *C. neoformans* total cellular DNA was purified as described previously (22). Restriction fragments generated by digesting total cellular DNA samples to completion with the restriction enzyme *Eco*RI (Promega Corp., Madison, Wis.) were separated by electrophoresis through 0.8% (wt/vol) agarose gels and transferred onto nylon membrane filters (MSI, Westboro, Mass.) according to the method of Southern (21). The oligonucleotide probe (GGAT)₄ was end labelled with [γ -³²P]dATP (Amersham International Plc., Little Chalfont, Buckinghamshire, United Kingdom [$>5,000 \text{ Ci mmol}^{-1}$]) and T4 polynucleotide kinase (Promega Corp.) according to the manufacturer's instructions. Labelled (GGAT)₄ was hybridized to the digested DNA as described previously (24).

RAPD. PCRs were performed in a final volume of 25 μl containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 3.0 mM MgCl₂; 200 μM (each) dATP, dCTP, dTTP, and dGTP (Promega Corp.); 20 pM primer; 0.5 U of *Taq* DNA polymerase (Promega Corp.); and approximately 10 ng of *C. neoformans* total genomic DNA. The following oligonucleotide primers were used: 1, 5'-GCGATCCCCA3'; 2, 5'(GATA)₄3'; and 3, 5'-AACGCGCAAC3'. Amplification reactions were performed in a Perkin-Elmer Cetus DNA thermal cycler under the following conditions: 94°C for 5 min, 36°C for 5 min, and 72°C for 5 min for 4 cycles followed by 30 cycles at 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. This was followed by an incubation period of 72°C for 10 min. After amplification, 20- μl aliquots of the reaction mixtures were electrophoresed in 1.5% (wt/vol) agarose gels, and the amplified products were visualized under UV light after being stained with ethidium bromide.

RESULTS

Oligonucleotide fingerprinting of *C. neoformans* isolates. Total cellular DNA was purified from a single isolate of *C. neoformans* recovered in each case from CSF, skin, and blood samples obtained from two patients with meningitis and from CSF cultures recovered from another four patients suffering from meningitis. Aliquots of DNA from these isolates were digested to completion with *Eco*RI, and the resulting fragments were separated by agarose gel electrophoresis and transferred to nylon membrane filters prior to hybridization analysis with the ³²P-labelled oligonucleotide probe (GGAT)₄. The fingerprints generated from isolates recovered from different anatomical sites in the same patient were found to be indistinguishable. However, the fingerprints generated from isolates recovered from separate patients were each found to be very

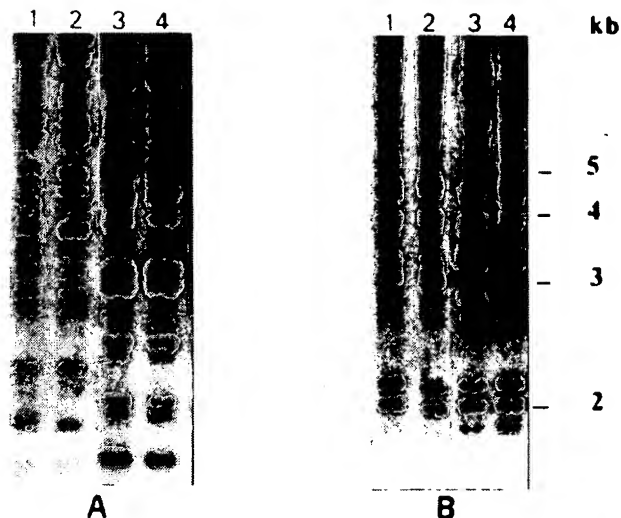


FIG. 1. Autoradiogram of *Eco*RI-digested total cellular DNA from single-colony isolates of *C. neoformans* recovered from CSF specimens from patients A and B during two successive episodes of meningitis and hybridized with the oligonucleotide probe (GGAT)₄. Lanes 1 and 2 and 3 and 4 show fingerprint profiles generated from two single-colony isolates recovered from the same CSF sample during the initial and recurrent episodes of infection, respectively. (A) Lanes 1 to 4 show profiles generated from isolates A₁1, A₁2, A₁₁1, and A₁₁2, respectively. (B) Lanes 1 to 4 show profiles generated from isolates B₁1, B₁2, B₁₁1, and B₁₁2, respectively. The relative positions of molecular size standards are indicated to the right.

different (data not shown), indicating that each patient was infected with a unique strain of *C. neoformans*.

Total cellular DNA was purified from three single-colony isolates from the initial and recurrent isolate collections recovered from two AIDS patients (A and B), each of whom experienced two successive episodes of meningitis, and DNA fingerprints were generated as described above. In the case of patient A, the hybridization profiles obtained with the three isolates recovered during the initial episode of meningitis (data for isolates A₁1 and A₁2 are shown in Fig. 1A, lanes 1 and 2) were identical to each other but were clearly significantly different from the corresponding profiles obtained with the three isolates (also identical to each other) recovered during the recurrent episode of infection (data for isolates A₁₁1 and A₁₁2 are shown in Fig. 1A, lanes 3 and 4). In contrast, the hybridization patterns obtained with both sets of isolates recovered from patient B were found to be very similar, but some band differences were evident. For example, there was a hybridization band with a size of approximately 3 kb, which was present in the profiles of the three isolates from the initial episode of infection (data for isolates B₁1 and B₁2 are shown in Fig. 1B, lanes 1 and 2) but was absent in the corresponding profiles of the three isolates from the recurrent episode (data for isolates B₁₁1 and B₁₁2 are shown in Fig. 1B, lanes 3 and 4). Direct visual analysis of ethidium bromide-stained agarose gels containing separated *Eco*RI-generated fragments of genomic DNA from the isolates concerned showed that the restriction fragment length polymorphism patterns of the recurrent isolates from patient B lacked a heavily stained band, also approximately 3 kb in size, that was present in the restriction fragment length polymorphism patterns of the three isolates from the initial episode of infection (data not shown). Furthermore, an additional hybridization band with a size of approximately 2.8 kb was present in the profile of isolate B₁₁1 which was not present in the profile of isolate B₁₁2 (Fig. 1B, lanes 3 and 4).

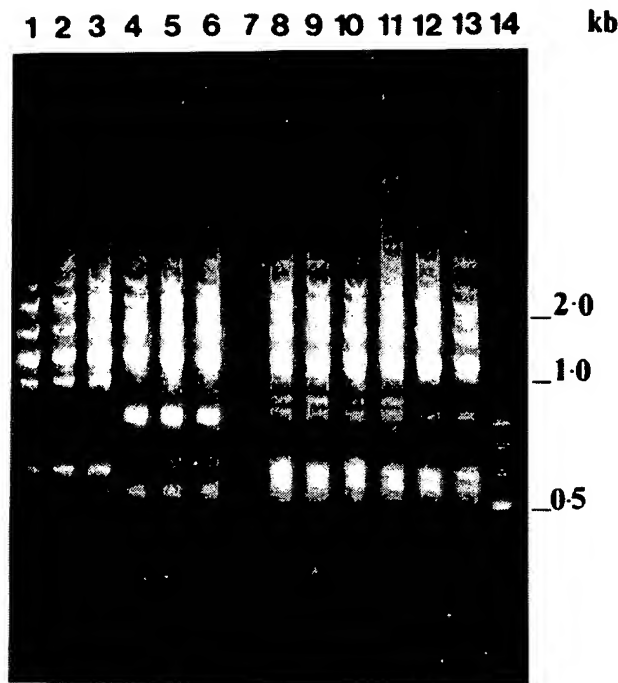


FIG. 2. RAPD products from single-colony isolates of *C. neoformans* recovered from CSF specimens from patients A and B during two successive episodes of meningitis and amplified with oligonucleotide primer 1 (5'-GGCATCCCC A3'). Lanes 1 to 3 (isolates A_I1 to 3) and 8 to 10 (isolates B_I1 to 3) show RAPD profiles generated from three single-colony isolates recovered from the same CSF sample from patients A and B, respectively, during the initial episodes of infection. Lanes 4 to 6 (isolates A_{II}1 to 3) and 11 to 13 (isolates B_{II}1 to 3) show RAPD profiles generated from three single-colony isolates recovered from the same CSF sample from patients A and B, respectively, during recurrent episodes of infection. Lane 14, 100-bp DNA ladder size standards (Promega).

The fingerprint profiles of the isolates described above were found to be stable after repeated subculture (minimum of eight times) of the isolates over a 6-month period. During this time, total cellular DNA was prepared from each isolate on three separate occasions, and in each instance, fingerprint profiles identical to those shown in Fig. 1 were obtained with (GGAT)₄.

RAPD analysis of recurrent isolates. The RAPD profiles generated with primer 1 and target DNA from three single-colony isolates from the same clinical specimen examined from the initial episode of infection in patient A (A_I1, A_I2, and A_I3) were identical to each other (Fig. 2, lanes 1 to 3). Similarly, the corresponding profiles obtained with the target DNA from an additional three single-colony isolates from the same clinical specimen recovered during the recurrent episode of infection (A_{II}1, A_{II}2, and A_{II}3 [Fig. 2, lanes 4 to 6]) were also found to be identical to each other. However, the patterns obtained from both sets of isolates were totally distinct. In contrast, the RAPD patterns generated with the three isolates examined from both the initial and recurrent episodes of meningitis in patient B (B_I1, B_I2, B_I3, B_{II}1, B_{II}2, and B_{II}3) were all found to be identical (Fig. 2, lanes 8 to 13). Additional RAPD experiments with the same target DNA as that used in the experiments described above and primers 2 and 3 also generated distinct profiles for the three A_I and the three A_{II} isolates and were unable to discriminate between the three B_I and the three B_{II} isolates. RAPD fingerprint profiles were also found to be reproducible after repeated subculture of the isolates with target DNA prepared on three separate occasions.

Analysis of multiple single-colony isolates. In order to investigate the possibility that patients A and B may have been infected with more than one strain of *C. neoformans* during both episodes of meningitis, total cellular DNA was prepared from an additional 13 single-colony isolates (9 in the case of the initial episode of infection in patient B) recovered, in each case, from the same clinical specimens as the isolates used in the experiments described above, and then hybridization fingerprints were generated with (GGAT)₄. For both patients, only a single fingerprint pattern was found for each group of single-colony isolates examined. As in the results described above, the patterns of the 13 additional A_I isolates differed considerably from those of the 13 additional A_{II} isolates, while those of the 9 additional B_I isolates were almost identical to those of the 13 additional B_{II} isolates. However, whereas the A_{II} isolates all yielded identical fingerprint patterns (fingerprints from 14 isolates are shown in Fig. 3a), the corresponding profiles of the A_I, B_I, and B_{II} isolate collections, respectively, although essentially homogeneous, showed numerous subtle but distinct minor polymorphisms. An example of the profiles obtained with the B_{II} isolate collection is shown in Fig. 3b.

RAPD fingerprints of the additional single-colony isolates from each isolate collection from patients A and B were also generated with primer 2 and primer 3, respectively. The results confirmed the findings obtained in the experiments described above with three single-colony isolates from each isolate collection. However, minor polymorphisms were evident in some of the RAPD profiles of all four single-colony isolate collections, including one single-colony isolate from the recurrent episode of infection in patient A (Fig. 4).

Fluconazole susceptibility of *C. neoformans* isolates. The fluconazole susceptibilities of isolates 1 to 3 from the initial and recurrent isolate collections for both patients A and B were determined by broth microdilution. Isolates A_I1 to 3, B_I1 to 3, and B_{II}1 to 3 yielded an IC₈₀ of 32 µg ml⁻¹, whereas isolates A_{II}1 to 3 yielded an IC₈₀ of 8 µg ml⁻¹.

DISCUSSION

Only a small number of studies have focused on the relationship between isolates of *C. neoformans* from successive episodes of meningitis in individual patients (8, 17, 22, 23). Some of these studies have indicated that relapse of cryptococcal meningitis is due to the persistence of the originally infecting strain (17, 22, 23), whereas another study has shown that in two separate individuals, recurrence of infection was apparently due to reinfection with a novel strain (8). In addition, the latter study provided evidence that one patient was infected with more than one *C. neoformans* strain during a single episode of meningitis. The present study was undertaken to confirm these findings by analysis of multiple *C. neoformans* single-colony isolates recovered from the same clinical specimen obtained from two patients during each of two successive episodes of disease. Fingerprint analysis of genomic DNA with the microsatellite sequence-containing oligonucleotide probe (GGAT)₄ and RAPD analysis with three separate oligonucleotide primers showed that the six patients included in the study were each infected by different *C. neoformans* strains. This is in agreement with earlier studies which described the genetic heterogeneity of serotype A *C. neoformans* strains (3, 7, 8, 17, 26, 27). In addition, in the case of the two patients from which *C. neoformans* isolates were recovered from specimens taken from different anatomical sites during the same episode of meningitis, all of the isolates from the same individual yielded the same fingerprint pattern.

Two of the six patients under study (A and B) suffered

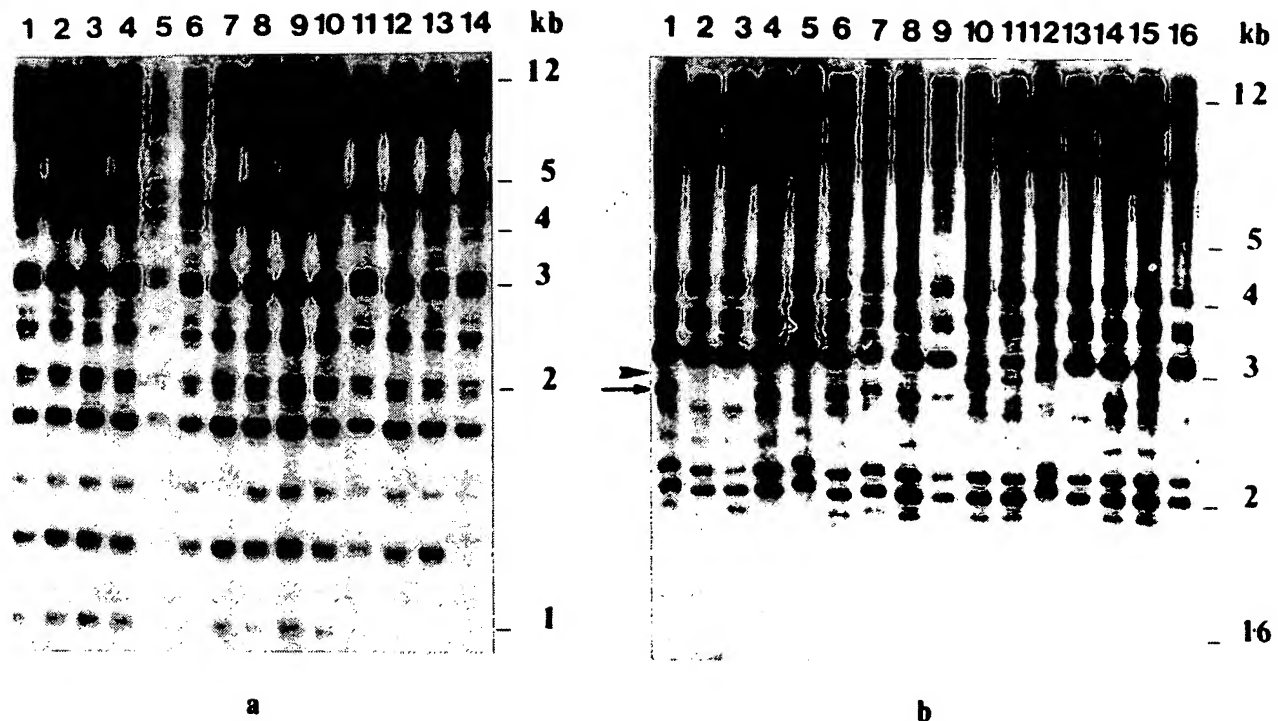


FIG. 3. Autoradiograms of *Eco*RI-digested total cellular DNA from *C. neoformans* single-colony isolates recovered from the same CSF specimen in the case of patients A and B, respectively, during the recurrent episode of meningitis after hybridization analysis with the oligonucleotide probe (GGAT)₄. (a) Profiles shown in lanes 1 to 14 were from single-colony isolates A₁₁1 to A₁₁14, respectively. (b) Profiles shown in lanes 1 to 16 were from single-colony isolates B₁₁1 to B₁₁16, respectively. The arrow and arrowhead shown to the left of panel b indicate the relative positions of polymorphic hybridization bands with sizes of approximately 2.6 and 2.9 kb present in isolate profiles shown in lanes 1, 4, 6, 7, 8, 9, 14, and 15 and lanes 10, 11, 12, and 15, respectively.

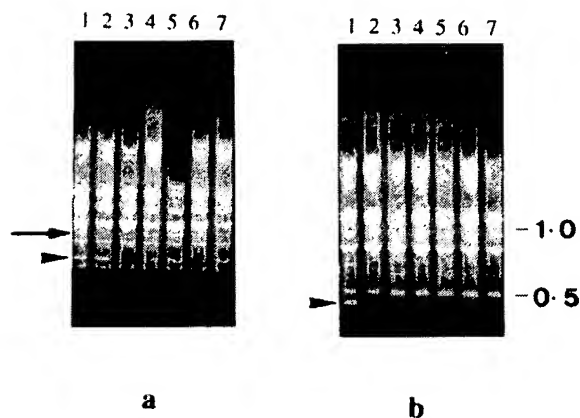


FIG. 4. Amplified RAPD products from single-colony isolates of *C. neoformans* recovered from CSF specimens from patient A during the initial and recurrent episodes of infection. (a) Profiles shown in lanes 1 to 7 were from single-colony isolates (A₁₁5 to A₁₁11) from the same specimen recovered during the initial episode of infection. (b) Profiles shown in lanes 1 to 7 were from single-colony isolates (A₁₁1 to A₁₁7) from the same specimen recovered during the recurrent episode of infection. The positions of the molecular size reference markers indicated to the right are in kilobase pairs. The arrowhead and arrow shown to the left of panel a indicate the relative positions of polymorphic bands with sizes of approximately 0.7 and 0.9 kb present in isolate profiles shown in lanes 1, 2, 4, 5, and 7 and 4, 5, 6, and 7, respectively. The arrowhead to the left of panel b indicates the position of a single polymorphic band with a size of approximately 0.45 kb present only in lane 1.

relapses in infection within 6 months of clinical resolution of the symptoms after therapy. Preliminary experiments were performed with three single-colony isolates recovered from CSF specimens from patients A and B during each episode of disease by both DNA fingerprinting techniques (Fig. 1 and 2). The results showed that the (GGAT)₄-generated hybridization patterns obtained with the three single-colony isolates from the initial episode of meningitis in patient A, although identical to each other, were significantly different from the corresponding profiles of the three single-colony isolates recovered during the recurrent episode of disease in the same patient. In fact, the two sets of profiles, each of which contained ≥ 15 clearly resolved hybridization bands, shared no bands in common, strongly suggesting that patient A was infected with unrelated *C. neoformans* strains during each episode of meningitis. In contrast, the hybridization patterns obtained with the three single-colony isolates from both the initial and recurrent episodes of infection in patient B were very similar, with the majority (~75%) of bands shared in common. These results suggested that the same strain of *C. neoformans* was responsible for both episodes of disease. This also suggested that the recurrent episode of infection was due to persistence of the strain responsible for the initial episode in patient B. However, the possibility that patient B was reinfected with the same strain from an environmental source cannot be discounted.

Fluconazole susceptibility data obtained from three single-colony isolates from each episode of meningitis in both patients A and B showed that isolates from the recurrent episode in patient A had a fourfold lower ($8 \mu\text{g ml}^{-1}$) fluconazole IC₈₀ than that obtained with isolates from the initial episode ($32 \mu\text{g ml}^{-1}$). In contrast, all six isolates tested from patient B yielded

the same fluconazole IC_{80} ($32 \mu\text{g ml}^{-1}$). These results strengthen the conclusions derived by fingerprinting analysis that the recurrent episode of meningitis in patient A was caused by a novel strain, while both episodes in patient B were caused by the same strain.

On the basis of the results described above, it was still possible that both patients A and B were infected with two or more strains of *C. neoformans* during each episode of disease and that this was not reflected in the single-colony isolates tested from primary isolation plates. If two strains were present during a particular episode of infection in different relative abundance, the chance of detecting a particular strain would directly reflect the number of single-colony isolates tested from the primary isolation plates. A strain present in low abundance relative to a second strain during a particular episode of disease in a given patient might not be detected if only a few colonies were sampled. A change in the relative abundance of the two strains during a subsequent episode of disease, assuming that both strains persisted between episodes, could lead to the conclusion that the recurrent episode was due to a novel strain. To unequivocally determine the presence of multiple strains during a specific episode of infection would require analysis of all of the *C. neoformans* colonies recovered on primary isolation. However, from a routine perspective, this would clearly not be practical for logistical reasons. To improve the chances of detecting the presence of more than one *C. neoformans* strain from each of the specimens obtained during both episodes of infection in patient A and from the recurrent episode of infection in patient B, a total of 16 single-colony isolates from the primary isolation plates were analyzed (i.e., an additional 13 single-colony isolates from each specimen). This represented >50% of the total number of *C. neoformans* colonies recovered from the original specimens in each case. In the case of the initial episode of infection in patient B, all 12 (i.e., 100%) of the *C. neoformans* CFU recovered on primary isolation were analyzed. Fingerprinting analysis of the additional single-colony isolates from the initial and recurrent specimens from patients A and B confirmed the preliminary findings described above with each of three single-colony isolates from each episode of infection. The very close similarity of the fingerprint patterns obtained with the initial and recurrent isolate collections from patient B makes it unlikely that this patient was infected by more than one strain of *C. neoformans* during each episode of meningitis and suggests that both disease episodes were caused by the same strain. Furthermore, although the fingerprint patterns obtained with the single-colony isolate collections from the initial and recurrent episodes of disease in patient A were totally different from each other, the profiles obtained with the 16 isolates comprising each collection were remarkably homogeneous, apart from minor polymorphisms evident in patterns from isolates from the initial episode. These findings and the confirmatory data obtained by RAPD analysis provided strong evidence that patient A was infected with a single strain of *C. neoformans* during each episode of disease and that a different strain was responsible for each episode.

As far as we are aware, the present work and another recent report from our laboratories (8) are the only studies which have indicated that recurrent episodes of meningitis in the same patient can be caused by different strains of *C. neoformans*. The only other published studies on this subject indicate that recurrent episodes of meningitis in the same patient are due to persistence of the same *C. neoformans* strain responsible for the initial episode of disease (17, 22, 23). There are several possible reasons for the disparity between our results and those of previous studies, including different patient man-

agement and treatment regimens. However, the use of different fingerprinting techniques in the various studies to distinguish between isolates and their relative discriminatory powers is probably more important. Each of the fingerprinting probes employed to date recognizes different genetic markers and is likely to have a different discriminatory ability.

Failure to detect minor polymorphisms in the single-colony isolate collection from the recurrent episode of infection in patient A by fingerprinting with the (GGAT)₄ probe was surprising, given that they were readily detectable in both isolate collections from patient B and in the initial isolate collection from patient A (Fig. 4a, lane 1). However, minor polymorphisms were evident in the RAPD profiles of one of the 16 single-colony isolates from the recurrent episode of meningitis in patient A. It is possible that some strains of *C. neoformans* are inherently more unstable or genetically pleomorphic and thus are more prone to generate polymorphisms than others. In order to investigate whether the polymorphisms were due to genetic instability in vitro, three of the single-colony isolates from each isolate collection recovered from patients A and B were subcultured at least eight times over a 6-month period, and fingerprinting experiments were repeated on three separate occasions with freshly prepared total cellular DNA. The results demonstrated that the fingerprint profiles of the single-colony isolates were reproducible. Furthermore, storage of isolates for a period of at least 6 months at -20°C did not detectably affect the fingerprint profiles. All of these results demonstrated that the generation of polymorphisms was not due to genetic instability in vitro; the generation of polymorphisms is more likely to be a response to unfavorable environmental conditions in vivo. Similar polymorphisms in *Candida albicans* DNA fingerprint profiles have recently been described and have been attributed to the occurrence of microevolution (12).

It has been suggested that *C. neoformans* strains only rarely reproduce sexually and that clinical populations of the organism are usually clonal in origin (5, 14). In the absence of genetic exchange through sexual reproduction, microevolution could conceivably confer a selective advantage under unfavorable environmental conditions.

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The emergence of a highly transmissible lineage of *cbl*⁺ *Pseudomonas* (*Burkholderia*) *cepacia* causing CF centre epidemics in North America and Britain

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The rapid increase in *Pseudomonas* (*Burkholderia*) *cepacia* infection in cystic fibrosis (CF) patients suggests epidemic transmission, but the degree of transmissibility remains controversial as conflicting conclusions have been drawn from studies at different CF centres. This report provides the first DNA sequence-based documentation of a divergent evolutionary lineage of *P. cepacia* associated with CF centre epidemics in North America (Toronto) and Europe (Edinburgh). The involved epidemic clone encoded and expressed novel cable (Cbl) pili that bind to CF mucin. The sequence of the *cblA* pilin subunit gene carried by the epidemic isolates proved to be invariant. Although it remains to be determined how many distinct, highly transmissible lineages exist, our results provide both a DNA sequence and chromosomal fingerprint that can be used to screen for one such particularly infectious, transatlantic clone.

Pseudomonas aeruginosa accounts for up to 90% of morbidity and mortality in patients with cystic fibrosis (CF) following persistent infection over a period of years. However, during the last decade, as many as 40% of the patients in some CF centres¹⁻⁴ have also become infected with *Pseudomonas* (*Burkholderia*) *cepacia*. About 20% of the latter die from bacteremia, or aggressive pulmonary infection over a few months^{5,6}. While the significant increase in *P. cepacia* infection suggests epidemic spread^{3,6-8}, the source and transmissibility of *P. cepacia* remains controversial⁹. Nonetheless, given the potentially grave consequence of *P. cepacia* infection, stringent infection control policies have been adopted, many CF camps in North America have been closed and all but one lung transplant centre have ceased to accept *P. cepacia*-infected CF patients as transplant candidates.

The epidemiology of *P. cepacia* infection has been examined by both ribotyping^{1,9} and pulsed-field gel electrophoresis (PFGE)-based resolution of chromosomal macro-restriction fragment length polymorphisms (RFLPs)^{10,20}. Comprehensive studies applying both methods generated two very different conclusions regarding clonality, persistence, and transmissibility. One study in the United Kingdom (Western General Hospital, Edinburgh), found a clonal relationship among isolates from 13 patients over six years³. In contrast, during an eight-year period at a US CF centre (University of North Carolina (UNC) Hospitals, Chapel Hill), not a single identical or closely related strain was found among 23 infected clinic and lung transplant patients⁹. Serial isolate analysis further confirmed this picture, typically demonstrating persistent infection by a single strain.

There also existed an isolate collection from another CF centre (Hospital for Sick Children, Toronto), where there was anecdotal evidence for an epidemic of *P. cepacia*. Although the isolates were not characterized for genetic relatedness, they had been uniformly resolved to express peritrichous, giant cable (Cbl)-like pili that specifically bound to CF mucin and airway epithelial cells^{4,11-13}. As the *cblA* pilin subunit gene encoded by all 15 of the Toronto isolates was the first such gene characterized for *P. cepacia*, a subsequent hybridization-based survey for the presence of *cblA* was carried out on multiple isolates from eight other CF centres in the United States and Europe as well as clinical and environmental strains. All of these isolates were *cblA*⁺ except for one isolate from a CF centre in Jackson, Mississippi.

Studies described in this article examine the genetic relatedness of *cblA*⁺ and *cblA*⁻ strains. The evolutionary picture generated indicates the emergence of a highly transmissible lineage, seemingly adapted for efficient transmission in the CF population. The resolved genetic markers uniquely associated with this lineage may be used to rapidly identify its presence and are therefore of immediate practical importance to CF centres in both Europe and North America.

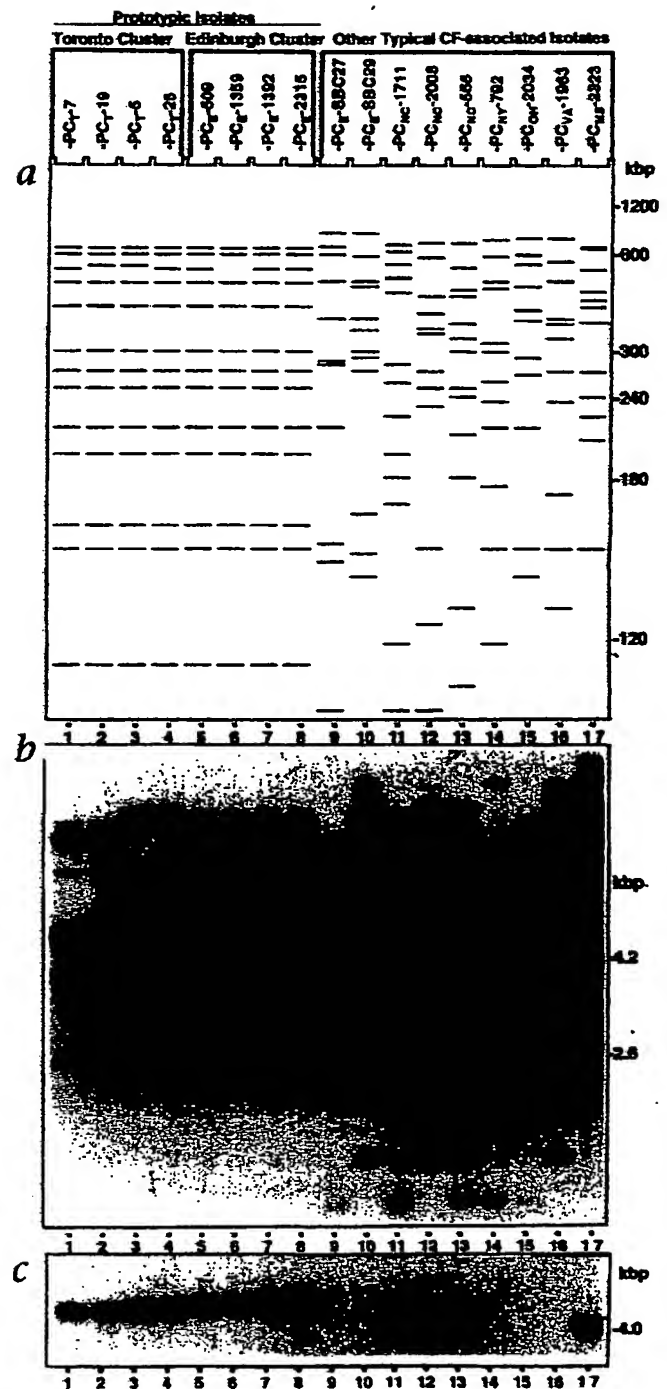
Genetic relatedness of *P. cepacia* isolates

The observations described above led us to characterize the epidemiological relatedness of the 15 Toronto isolates expressing mucin-binding Cbl pili. To investigate the genetic relationship between these isolates and those found elsewhere, we included clinical and environmental isolates as well as 78 strains from the

Fig. 1 RFLPs of 17 *P. cepacia* isolates cited in the text and Methods sections. Lane order for the 17 isolates is maintained in the three parts of the figure. Isolate numbers of examined strains appear at the top of the figure immediately above each lane. Subscript letters preceding isolate number indicate CF centre from which *P. cepacia* (PC) strain was isolated: PC_{Ed}, Edinburgh, Scotland; PC_{Ms}, Jackson, Mississippi; PC_{Ch}, Chapel Hill, North Carolina; PC_{NY}, New York, New York; PC_{OH}, Cleveland, Ohio; PC_T, Toronto, Canada; and PC_{NF}, Norfolk, Virginia. *a*, PFGE-resolved, chromosomal *SpeI* macro-RFLPs. As described previously⁹, samples were prepared and restriction fragments separated by pulsed-field gel electrophoresis with a CHEF Mapper system (Bio-Rad) through 1% agarose using a field strength of 6 V/cm and an initial and final pulse time of 1.2 s and 54 s, respectively. Fragment sizes were determined using a λ concatenate ladder (not shown). Bar-code format translation of chromosomal fingerprint profiles was made using a Macintosh Quadra 950 running Gene Construction Kit (Textco). Fragments below 100 kbp are not shown. In the latter range, Toronto and Edinburgh isolates displayed in lanes 1–8 had two identical fragments (60 kbp and 48 kbp). Other isolates (lanes 9–17) had polymorphic sets of three to six fragments in this lower range. *b*, *rm* (ribosomal RNA operon) *EcoRI* RFLPs. Southern blot hybridization methods were as we described previously²⁶ using a ³²P-labelled *rmB* probe spanning the entire *rmB* operon of *E. coli* K12. *c*, *cbIA* hybridization analysis of *EcoRI*-generated RFLPs. This was accomplished by stripping bound *rm* probe from the membrane used in Fig. 1*b* followed by hybridization with a previously described *cbIA* gene probe⁴ using standard methods^{26,27}.

seven other CF centres cited above that were *cbIA*⁺. At this time the report of RFLP-identical *P. cepacia* isolates transmitted among patients at an Edinburgh CF centre appeared, and we obtained the involved strains³ to include in this phylogenetic characterization (Fig. 1*a*, *b*). Profiles in lanes 9–17 of both *a* and *b* of Fig. 1 depict typical polymorphic patterns resolved for isolates from different CF centres. For these isolates mean D (Dice coefficient of similarity)²⁸ for any pair by PFGE-resolved chromosomal macro-RFLP profile was 0.14 ± 0.07 (Fig. 1*a*), a level of diversity not significantly different from that found previously among eight independently isolated American Type Culture Collection (ATCC) environmental and clinical control isolates⁹. A similar degree of chromosomal RFLP variability was found between the other CF-associated isolates from the seven CF centres (results not shown), confirming that these are epidemiologically distinct strains with RFLP variability not significantly different from that of the random collection of ATCC strains ($0.1 > P > 0.05$). Despite the lower discriminatory power of ribotyping⁹, a similar degree of phylogenetic relationship among these CF-associated isolates is apparent in Fig. 1*b*.

The heterogeneity of the RFLP profiles of the isolates from the seven CF centres (for example, lanes 9–17, Fig. 1*a*, *b*) is similar to that described in a previous study involving multiple isolates from 23 patients at the UNC Hospitals CF centre⁹. This degree of variability contrasts markedly with the two closely related, conserved RFLP patterns found for the 15 *cbIA*-encoding Toronto CF centre isolates (lanes 1–4, Fig. 1*a*, *b*). Here, by examining both PFGE and ribotype RFLP profiles, the coefficient of similarity among the Toronto isolates proved to be very high, with PFGE $D = 0.95 \pm 0.03$ and ribosomal RNA operons (*rm*) $D = 0.87 \pm 0.09$. This contrasted with (*a*) the mean D value among isolates from the other seven CF centres, which was very low (for example, lanes 9–17, Fig. 1*a*, *b*), and (*b*) the mean D between the Toronto isolates and the other CF centre iso-



lates, which was also very low: PFGE $D = 0.20 \pm 0.07$, *rm* $D = 0.39 \pm 0.09$. These findings strongly suggest that all 15 of the Toronto CF centre isolates were members of a unique lineage associated with an epidemic.

Displayed in lanes 5–8 of Fig. 1*a* and *b* are *P. cepacia* PFGE and ribotype RFLP profiles of isolates from CF patients at the Edinburgh CF centre³. The RFLP profiles displayed in Fig. 1*a* and *b* also indicate the presence of an epidemic clone, as D for any analysed pair by either type of RFLP profile was very high (PFGE $D = 0.98 \pm 0.02$, *rm* $D = 1.0$). Further, pairwise comparison of the Edinburgh strains to the closely related Toronto CF centre strains (lanes 1–4 of Fig. 1*a*, *b*) likewise produces robust D values (PFGE $D = 0.97 \pm 0.03$, *rm* $D = 0.90 \pm 0.04$), strongly suggesting that the

Edinburgh and Toronto isolates are members of the same unique lineage despite the Atlantic Ocean barrier.

Phylogenetic relationships of *P. cepacia* isolates

Based on ribosomal RNA operon (*rrn*) RFLP profiles, phylogenetic relationships of the 133 isolates described above and in the Methods section were determined with the neighbour-joining method¹⁵. Confidence intervals on the tree topology were estimated by bootstrapping analysis¹⁶ (Fig. 2). The resultant phylogenetic tree indicates that the cluster of Toronto and Edinburgh isolates comprises a single, clonally related lineage. The remaining, independently isolated strains from other CF centres are as distantly related to one another as they are to either the Toronto/Edinburgh clusters or the independently isolated non-CF clinical and environmental strains.

Cbl phenotype and genotype of Edinburgh CF isolates

We then examined the epidemic Edinburgh isolates to see if, as do the Toronto strains⁴, they expressed Cbl pili and encoded the *cblA* gene. Phenotypic survey using electron microscopy revealed that these highly transmissible strains expressed appendage pili that were structurally equivalent to those expressed by all of the *cblA*⁺ Toronto isolates^{4,13} (Fig. 3). Genotypic survey was carried out by stripping *rrn*-probe from an *Eco*RI chromosomal digest membrane (Fig. 1b) followed by hybridization with a *cblA* probe. The highly transmissible Edinburgh isolates as well as the closely related Toronto clones encode *cblA* (Fig. 1c, lanes 1–8).

cblA gene sequence-based test of clonality

Because the implications of our studies have the potential to in-

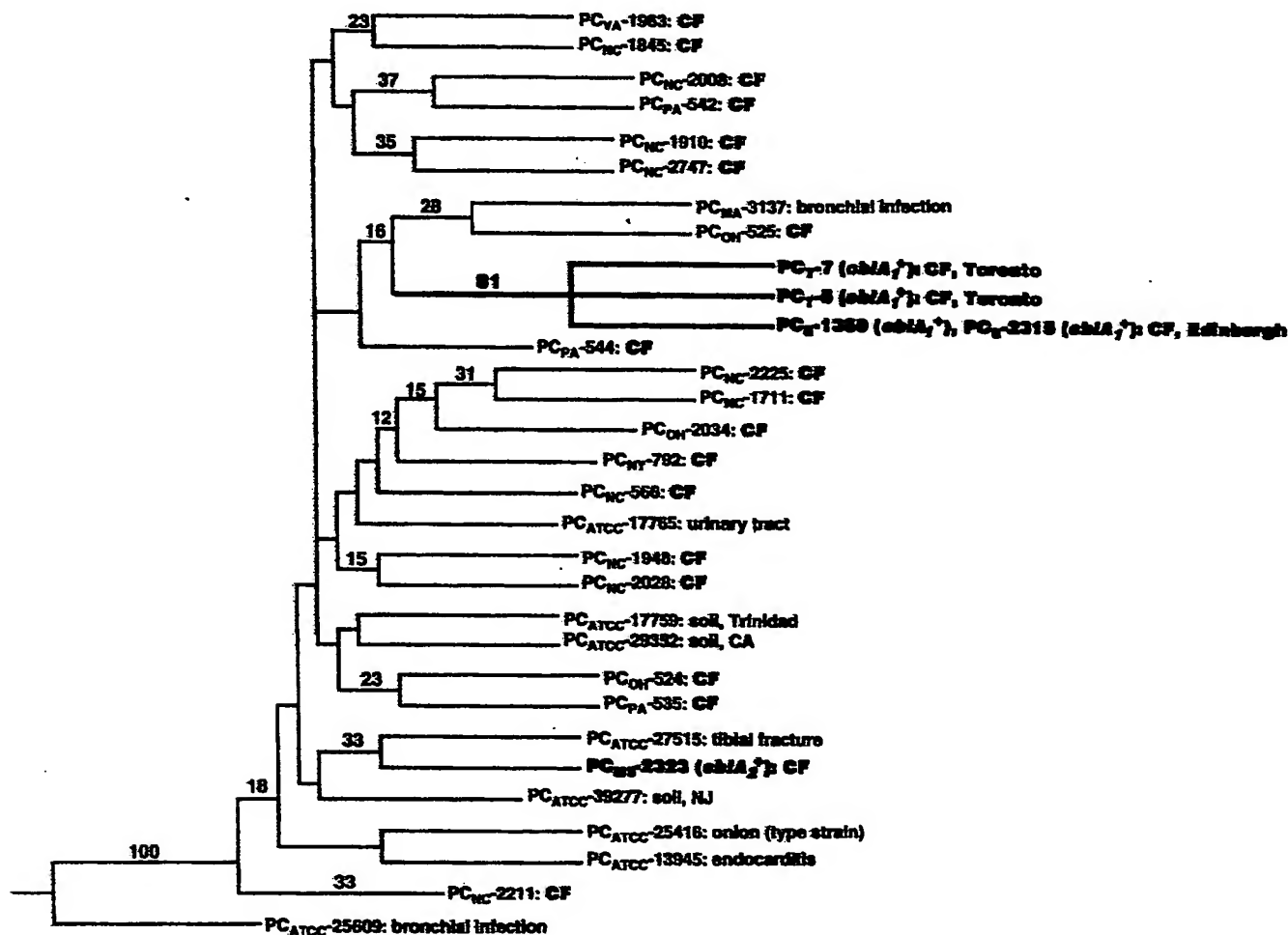


Fig. 2 *rrn*-RFLP-based phylogenetic tree of representative isolates from patients at seven CF centres in North America (Chapel Hill, North Carolina; Jackson, Mississippi; Norfolk, Virginia; Cleveland, Ohio; Philadelphia, Pennsylvania; New York, New York; Toronto, Ontario) and the United Kingdom (Edinburgh) plus environmental and clinical (non-CF) sources. All cited isolates are described in the text and in the Methods section. Indicated isolate number is followed by source (CF, environmental or clinical). *cblA*_T⁺, *cblA*_E⁺: isolate(s) that encode the *cblA* gene (Fig. 1c) and express adhesin Cbl pili (Fig. 3). *cblA*_T⁺, identical 501-bp sequence carried by Toronto and Edinburgh CF centre isolates (Fig. 4); *cblA*_T⁺, polymorphic 501-bp sequence carried by Jackson, Mississippi, CF centre isolate PC_{MA}-2323 (Fig. 4). Numbered above each branch indicates the percentage of times each was joined together under bootstrap analysis¹⁶ (confidence intervals less than ten have been omitted for clarity). The lineages included in this tree are representative of the larger sample of isolates collected. Multiple CF patient serial isolates of an identical *rrn* RFLP profile have not been included as they do not affect the tree topology. However, multiple isolates from Toronto (PC_T-5, PC_T-7) and Edinburgh (PC_E-1359, PC_E-2315) CF centres are noted because further analysis by DNA sequence revealed that the *cblA* genes encoded by these four isolates are identical (Fig. 4). The remaining 13 and 11 isolates, respectively, from each of these two CF centres are members of the indicated epidemic lineage based on 100% correlation of their *rrn* RFLP profiles with those of the prototypic patterns of the Toronto/Edinburgh isolates shown in Fig. 1b.

fluence directly clinical management of some 70,000 CF patients in Europe and North America, we used DNA sequence analysis to test the RFLP-based conclusions that isolates from Toronto and Edinburgh were clonal. Either of two classes of bacterial genes are typically sequenced for this purpose: slowly evolving, 'house-keeping' genes such as *putP* (proline permease)¹⁷ or, more rapidly evolving, antigen-encoding genes such as the flagellar filament gene (*flaC*) of *Salmonella typhimurium*¹⁸. Based on analogy to the latter, we chose the *cblA* pilin gene as it would probably be under antigenic selection, thus providing a more rigorous test for the clonality of strains. The possibility of antigenic variability being reflected in the *cblA* pilin gene sequence was suggested by our analyses of the Jackson, Mississippi, CF-associated strain PC_{MS}-2323, the sole *cblA*-positive isolate not associated with the epidemic clusters (lane 17, Fig. 1c). Variability in the encoded *cblA* of this isolate had been inferred by (1) ribotype and chromosomal macro-RFLP profiles indicating that PC_{MS}-2323 was only distantly related to the *cblA*-positive Toronto/Edinburgh isolates (lane 17 versus lanes 1–8 of Fig. 1a, b); (2) variation in *cblA*-encoded restriction fragment size from that observed in the Toronto/Edinburgh isolates (lane 17 versus lanes 1–8 of Fig. 1c) and (3) absence of agglutination by antibodies made against Cbl pili purified from *cblA*-positive Toronto CF centre isolates (data not shown).

Primers were synthesized from the *cblA* sequence of the Toronto isolate PC_T-7 (ref. 4) and used for polymerase chain reaction (PCR)-based amplification of the *cblA* gene from isolates to be characterized. Resultant PCR products were then cloned and sequenced (see Methods). Complete *cblA* sequences were thus obtained from isolates with the two slightly variant though closely related RFLP profiles typical of the 15 Toronto CF centre isolates (Fig. 1a, b, lanes 1–4), the two slightly variant though closely related RFLP profiles typical of the 13 Edinburgh CF centre isolates (Fig. 1a, b, lanes 5–8), and the significantly variant Jackson, Mississippi, CF-associated strain PC_{MS}-2323 (Fig. 1a, b, lane 17). Comparison of these five sequences indicates that the chromosomally encoded, 501-base pair (bp) *cblA* pilin subunit structural gene carried by the closely related isolates from the Toronto and Edinburgh CF centres was invariant in sequence. In contrast, the *cblA* gene encoded by the distantly related Jackson Mississippi strain PC_{MS}-2323 exhibited polymorphism at the sequence level, with changes in 60 bp of the 501-bp sequence (88% identity; see Fig. 4). The perfect conservation of the *cblA* pilin sequence among isolates from multiple patients over four years at the Toronto and Edinburgh centres is precisely what would be expected for epidemic transmission of a highly infectious clone. Likewise, the variant *cblA* encoded by the Mississippi CF centre isolate is in accord with that expected for a distantly related isolate (Fig. 2).

Discussion

P. cepacia varies in transmissibility

Consideration of these findings in the context of our previous studies on UNC CF centre isolates⁹ leads us to conclude that isolates of *P. cepacia* are not equally transmissible between CF patients, rather, there exists at least one significantly divergent, highly transmissible clonal lineage plus numerous moderately heterogeneous lineages of negligible transmissibility (see Fig. 2), and that the highly transmissible lineage identified is responsible for epidemics at North American and British CF centres. This was most likely due to an as yet unidentified transatlantic transmission related to joint summer camp attendance.



Fig. 3 Transmission electron micrograph of Toronto epidemic strain PC_T-7 expressing Cbl adhesin pili. High resolution was achieved with a JOEL 100 CX electron microscope as previously described¹⁹. Bar in lower right corner, 0.1 μ m. Taken from Goldstein *et al*¹⁹.

Relation of Toronto/Edinburgh clone to other epidemic strains

Based on *mm* RFLP profiles or anecdotal evidence, additional reports suggest the occurrence of *P. cepacia* transmission at CF centres in Philadelphia² and Cleveland¹⁰, respectively. We characterized strains involved with both of these putative outbreaks (see Methods section) and found that by neither ribotype or macro-chromosomal RFLPs profile did the prototypic RFLP fingerprints of the putatively epidemic strains from either centre appear similar to one another (mean D \leq 0.3), nor to the unique, highly transmissible lineage involved with the Toronto and Edinburgh CF patients (L.S., A.H., H. Zhou and R.G., unpublished data). Nonetheless, highly conserved RFLP profiles (mean D \geq 0.85) within the individual outbreaks did support a picture of epidemic transmission within each of the two centres. When these isolates were further characterized, hybridization-based survey for the presence of the *cblA* pilin gene proved negative for 35 of the involved strains (L.S., A.H., H. Zhou and R.G., unpublished data). These results suggest that there may exist *P. cepacia* lineages of high transmissibility other than the *cblA*⁺ clone that we have identified.

Emergence of clone for efficient infection of the CF lung

This study demonstrates the integral role of molecular epidemi-

ology and evolutionary biology in identifying newly emerging, highly transmissible microbial pathogens. The degree of divergence of the *cbIA*⁺ lineage suggests specific adaptive, evolutionary changes for efficient transmission in the CF population. A number of phenotypic observations support this hypothesis, such as the novel giant Cbl pili expressed, which have been found to promote adhesion-based colonization of the CF airway^{11,14}. New results provide further details, showing that *cbIA*⁺ isolates are significantly more adherent to human, primary culture *cfr*⁺ airway epithelial cells than *cbIA*⁻ strains, and that the *cbIA*⁺ isolates adhered to the epithelial apical surface as well as to the cilia (J. Yankaskas, P. Gilligan and R.G., unpublished observations), suggesting the potential for interference with the mucociliary transport system. Further, unlike non-epidemic *P. cepacia* isolates, clones from the Toronto/Edinburgh epidemics proved uniquely resistant to killing by *P. aeruginosa* isolates cultured from many different patients (C. Campanelli, A.H. and R.G., unpublished observations). Given that CF patients are most often infected with *P. aeruginosa* before superinfection by *P. cepacia*, this atypical resistance may contribute to the remarkable capacity of the *cbIA*⁺ lineage to be epidemically spread among the CF population.

From molecular genetics to clinical management

Although it remains to be determined how many other divergent, highly transmissible lineages of *P. cepacia* have evolved, these results provide both the first chromosomal RFLP fingerprint (Fig. 1a, lanes 1–8) and DNA sequence (Fig. 4) that can be used for precise identification of one such lineage (Fig. 2). Knowledge of specific, intragenic regions of the invariant Toronto/Edinburgh *cbIA* sequence allows for rapid, PCR- or oligo hybridization-based screening for this highly infectious lineage (L.S., A.H., H. Zhou and R.G., unpublished observations). The resolution of a chromosomal fingerprint unique to a particular epidemic lineage indicates that it should be possible to establish an epidemiological library of such profiles. Among the increasing population of CF patients infected with *P. cepacia*, prospective screening for the highly transmissible lineages will provide a rational basis for patient isolation and infection control policies. This is of immediate relevance to the clinical management of the CF population as such decisions impact dramatically on patients, their families and the health-care system.

Methods

Bacterial isolates. *Pseudomonas cepacia* (*n* = 133) isolates were obtained from the following sources: (1) 65 isolates from patients at the University of North Carolina (UNC) Cystic Fibrosis Centre (1985–93) including 17 clinic and 5 transplant patients, 4 of whom were infected transfers from other distant locations as described previously^{9,13}. Those cited in the figures include isolates PC_{NC}-566, PC_{NC}-1711, PC_{NC}-1845, PC_{NC}-1910, PC_{NC}-1948, PC_{NC}-2008, PC_{NC}-2028, PC_{NC}-2211, PC_{NC}-2225 and PC_{NC}-2747, each from different local clinic patients, and four isolates from infected transfer patients (PC_{NY}-792 from New York, New York, PC_{VA}-1963 from Norfolk, Virginia, PC_{OH}-2034 from Cleveland, Ohio, and the *cbIA*⁺ isolate PC_{MS}-2323 from Jackson, Mississippi); (2) 8 ATCC clinical and environmental isolates: human endocarditis PC_{ATCC}-13945, human urinary tract PC_{ATCC}-17765, human bronchial PC_{ATCC}-25609, human tibia fracture PC_{ATCC}-27515, forest soil (Trinidad) PC_{ATCC}-17759, onion PC_{ATCC}-25416 (the ATCC *P. cepacia* type strain), soil (California) PC_{ATCC}-29352, cornfield soil (New Jersey) PC_{ATCC}-39277, all as described previously^{9,13}; (3) 15 *cbIA*⁺ isolates from 15 CF centre clinic patients at the

Hospital for Sick Children (1987–88), Toronto. Those cited in the figures include isolates PC_r-5, PC_r-7, PC_r-19, PC_r-25 (refs 4, 13); (4) 2 isolates, PC_e-SBC27 and PC_e-SBC29, from two CF patients at Western General Hospital (Edinburgh, Scotland) that were not associated with epidemic transmission within this CF centre^{3,13}; (5) 4 isolates from clinic patients at Western General Hospital (1989–90), Edinburgh, all of which had been associated with epidemic transmission within this CF centre³. Those cited in the figures include isolates PC_e-509, PC_e-1359, PC_e-1392 and PC_e-2315; (6) 10 isolates from 10 patients at a Philadelphia, Pennsylvania, CF centre². Those cited in figures include isolates PC_m-535, PC_m-542 and PC_m-544; (7) 24 isolates from 24 patients at the Rainbow Babies and Children's Hospital, Cleveland, Ohio¹⁹, including cited isolate PC_{OH}-524, PC_{OH}-525 and PC_{OH}-2034; and (8) 5 bronchial isolates from 5 non-CF ventilator patients at Boston City Hospital (Massachusetts) including cited isolate PC_m-3137.

RFLP, amplification and sequencing analyses. PFGE, ribotype and *cbIA* hybridization methods are described in detail in recent publications cited in the text^{4,9,13}. Using previously described methods⁹, CsCl equilibrium density gradient purified chromosomal DNA²⁰ was isolated from the two prototypic Toronto epidemic isolates (PC_r-7 and PC_r-5), the two prototypic Edinburgh epidemic isolates (PC_e-2315 and PC_e-1359) and the single Jackson, Mississippi, CF centre isolate

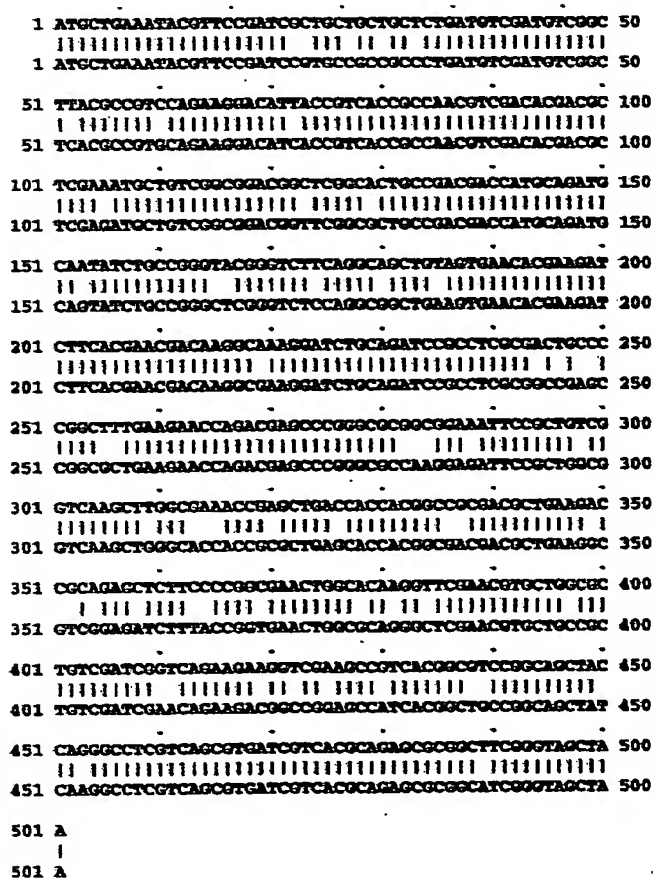


Fig. 4 Identical 501-bp sequence (topmost line) of the *cbIA* structural gene encoded by two prototypic Toronto epidemic isolates (PC_r-7 and PC_r-5) and two prototypic Edinburgh isolates (PC_e-2315 and PC_e-1359) compared to the variant *cbIA* sequence carried by the single Jackson, Mississippi, CF centre isolate PC_{MS}-2323 (lower line). See Methods section.

PC_{ms}-2323. From each of these *cbIA* probe-positive chromosomes, the *cbIA* gene was PCR amplified²¹ using a DNA thermocycler (Perkin-Elmer) with a GeneAmp PCR Core Reagents Kit (*ibid.*). Based on the previously determined sequence of the *cbIA* gene encoded by isolate PC_r-7 (ref. 4), sense and antisense primers used for these reactions were 5'-CCAAAGGACTAACCCA-3' and 5'-ACGCGATGTCCATCACA-3', respectively. PCR reactions were as follows: cycle one, 2 min at 94 °C, 2 min at 37 °C, 1 min at 72 °C. The remaining 29 cycles were: 1 min at 94 °C, 1 min at 45 °C, 1 min at 72 °C, followed by 7-min extension at 72 °C. PCR products were separated by electrophoresis through 0.8% agarose and for each a single band was observed with ethidium bromide staining. Bands were electroeluted into DEAE membrane (Schleicher & Schuell) and cloned with a TA Cloning Kit (Invitrogen). DNA sequences were determined by the Sanger dideoxy method²² with the same primers used for PCR amplification (above). Five PCR-amplified *cbIA* gene clones of PC_{ms}-2323 were generated, three of which were sequenced for confirmatory purpose, with no variation resolved.

Statistics. Standard criteria were used for comparing PFGE patterns²³. According to established criteria for *P. cepacia*²⁴, strains were assigned to the same ribotype when comparison of sizes of hybridizing fragments revealed three or fewer bands differing between the two patterns under comparison. Quantitative pairwise comparison of both types of RFLP patterns was accomplished using the Dice coefficient of similarity calculated as $D = 2n_{xy} / (n_x + n_y)$, where n_x is the total number of DNA fragments from strain X, n_y is the total number from strain Y, and n_{xy} the number of fragments identical in the two strains^{14,25}. The coefficient of similarity for two PFGE RFLPs $D \geq 0.90$ represents closely related strains, while unrelated strains have $D \leq 0.60$. Intervening values, remarkably, are rare⁹. For *rm* RFLPs, given that *P. cepacia* strains typically display 7–10 distinct hybridizing bands, the shared ribotype (above) would correspond to $D = 0.79$ to 0.85. Comparisons between mean values were performed by Student's *t*-test using a Systat program (Systat Inc.).

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Identification of IS1356, a New Insertion Sequence, and Its Association with IS402 in Epidemic Strains of *Burkholderia cepacia* Infecting Cystic Fibrosis Patients

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Burkholderia cepacia is now recognized as an important opportunistic pathogen in cystic fibrosis (CF) and other compromised patients. Epidemicity among CF patients has been attributed to at least one particularly infectious strain (strain ET12), and both genetic evidence and anecdotal evidence suggest that this strain, currently endemic in Ontario, and those causing an epidemic in the United Kingdom, are indeed the same. Our study was conducted to determine whether there was any association between the presence of various insertion sequence (IS) elements, the cable pilin subunit gene (*cblA*), electrophoretic type (ET), and ribotype (RT) in a collection of 97 clinical and 2 environmental isolates of *B. cepacia*. No apparent linkage was found for IS elements IS401, IS402, IS406, IS407, and IS408 with ET or RT. The *cblA* target, said to be a marker for high infectivity, was detected in 100% (38 of 38) of strains of *B. cepacia* ET12 and in a single strain of ET13 that differed in a single enzyme allele. A new IS, IS1356, identified during the investigation, was present in 71.7% of all isolates, and 50.7% of these isolates harbored IS1356 as a hybrid IS element inserted into IS402. IS1356 is 1,353 bp in length, and when it is inserted into IS402 it results in a 10-bp duplication at the site of insertion. IS1356 contains one major open reading frame of 1,260 bp coding for a putative transposase which has significant homology to IS*Rm3* in *Rhizobium meliloti* (59%) and to an undesigned IS element in *Corynebacterium diphtheriae* (49%). The IS402-IS1356 element was found exclusively in the epidemic strains from Ontario and the United Kingdom, being detected in 94.7% (36 of 38 isolates) of *B. cepacia* ET12 isolates. Of the two ET12 isolates found to be devoid of the IS402-IS1356 element, both contained IS1356 unassociated with IS402, one was temporally unrelated to the epidemic, and the other was from a CF patient in a geographic area remote from Ontario and the United Kingdom. It is evident that the IS402-IS1356 hybrid element, the *cblA* pilin subunit gene, and the allelic suite represented by multilocus enzyme electrophoretic type ET12 may provide useful markers for the epidemic, highly transmissible transatlantic strain isolated in Ontario and the United Kingdom.

Burkholderia cepacia is an aerobic gram-negative bacillus commonly found throughout the environment and as a phytopathogen causing soft rot in onions (1). Over the past decade, however, strains which cause opportunistic infections in humans, most notably in cystic fibrosis (CF) patients, have been encountered with increasing frequency, leading to an increase in morbidity and mortality (12, 38). Among non-CF patients extrapulmonary nosocomial infections have more recently been reported (21).

Although the mechanism of virulence of *B. cepacia* isolates has not been elucidated (19), isolates from CF patients have been shown to adhere to mucin (26) and buccal epithelial cells (27). There may also be a correlation between the source of *B. cepacia* isolates (e.g., environmental and CF-associated epidemic and nonepidemic isolates) and the particular class of pili expressed (9). The implications of being colonized with *B. cepacia* isolates are a growing concern in the CF patient community, and markers of strain virulence are eagerly sought. Enhanced transmissibility and virulence appear to be strain dependent, and epidemic lineages are being defined anecdot-

ally and genetically (10, 13, 17, 33-36). To date, studies have indicated cross-infection between patients (10, 17, 25, 31) and nosocomial acquisition (20) as important parameters of transmission.

In attempts to limit the spread of *B. cepacia* strains, many clinical centers now segregate colonized and noncolonized CF patients. This has proved to be successful but is limited by the social contacts between patients outside of the hospital setting that is the norm for CF patient groups, especially adults (10, 17, 31).

Many studies involving *B. cepacia* strains have focused on their truly extraordinary potential to metabolize a wide variety of organic compounds. It is currently thought that this metabolic versatility may, in part, be the result of the genomic complexity (24) and the large number of insertion sequence (IS) elements identified in *B. cepacia* strains (7, 15). IS elements have the ability to promote genomic rearrangement, recruit foreign genes, and cause insertional gene activation. Indeed, most of the IS elements in *B. cepacia* isolates have been identified by observing these features (16). The effect of IS elements on the genes with which they are associated is well documented (8), and it is conceivable that they may act genetically to increase the transmissibility and pathogenicity of certain strains of *B. cepacia*.

We originally identified the strains obtained by Govan et al. (10) in 1993 and ourselves (13, 25), from the United Kingdom

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TABLE 1. Oligonucleotide primers used to detect the various IS elements and *cblA* pilin subunit gene in *B. cepacia*

Target gene	Primer sequences	Amplicon size (bp)
IS402	A: 5'-CAA CCG AGA CTG AGG AGA TG-3' B: 5'-GCT GCT TGC CAA TCG CGC TC-3'	250
IS406	A: 5'-GAC GGT GGG TCT GAC GCC AT-3' B: 5'-AAG CCC TGA GTC CCT CGT CG-3'	450
IS407	A: 5'-TCA TCG GGT TTC TGA AGG AA-3' B: 5'-CGG AAG CGA GCT GCA CGG TC-3'	750
IS408	A: 5'-TTG AAG GAA GTC CTG CGA CT-3' B: 5'-TCG ACT TCG CCC AAT CCT TG-3'	370
IS1356	A: 5'-GGC CCT GAA GAA GGC GAT AT-3' B: 5'-TCC GGC GAC ACC TCG ATG CC-3'	327
<i>cblA</i> ^a	A: 5'-CCA AAG GAC TAA CCC A-3' B: 5'-ACG CGA TGT CCA TCA CA-3'	610

^a Primers were first described by Sajjan et al. (28).

and Canada, respectively, as having an identical enzyme electrophoretic allotype (electrophoretic type 12 [ET12]), the first direct evidence that the anecdotal association of Canadian *B. cepacia* strains currently endemic in Ontario and those causing an epidemic in the United Kingdom were the same. In the present study, the frequencies of occurrence of various IS elements were studied in our collection of clinical *B. cepacia* strains to determine if any relationship exists between these genetic modifiers and electrophoretic type (ET) or ribotype (RT). Additionally, a recent publication by Sun et al. (36) described the presence of novel cable pili in the epidemic clone described above, and our collection of strains was therefore also screened for the presence of the *cblA* pilin subunit gene.

MATERIALS AND METHODS

Bacterial strains, nucleic acid preparation, ribotyping, and multilocus enzyme electrophoresis. The collection of strains used in the investigation consisted of 99 isolates of *B. cepacia*, most of which were previously characterized for their ETs and RTs (13). Strains were grown overnight on Columbia blood agar base (Quelabs, Montreal, Quebec, Canada) at 37°C in 5% CO₂ prior to nucleic acid (NA) extraction. The isolates were originally obtained as isolates either from CF patients or from nosocomial outbreaks, and they were maintained in the culture collection of the Laboratory Centre for Disease Control, Ottawa, Ontario, Canada. Two of the strains investigated were American Type Culture Collection (ATCC) reference strains of environmental origin (ATCC 17759 and ATCC 25416). Procedures for NA purification, ribotyping, and multilocus enzyme electrophoresis were as described previously (13).

Oligonucleotide primers and PCR amplification. The sequences of the oligonucleotide primers designed to detect the various IS elements and the pilin subunit gene are summarized in Table 1 and are based on the published sequences for IS402 (6), IS406 and IS407 (41), IS408 (2), and *cblA* (36). Primers for the detection of IS1356 were designed on the basis of the sequence data acquired in the present investigation. All primers were synthesized on a 392 DNA-RNA Synthesizer (Applied Biosystems, Foster City, Calif.) by using standard phosphoramidite chemistry. Amplification was performed in a PE9600 thermocycler (Perkin-Elmer Cetus, Foster City, Calif.), with PCR mixtures containing 0.2 mg of NA per ml, 200 mM deoxynucleoside triphosphate, 1 mM (each) primer in the pair, 50 U of *Taq* polymerase (Boehringer Mannheim, Laval, Quebec, Canada) per ml, and 1× reaction buffer supplied by the manufacturer. Thermocycling

conditions consisted of an initial denaturation of 2 min at 94°C; this was followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. Following amplification, the samples were incubated at 72°C for 10 min and were then cooled to 4°C. Amplicons were detected by electrophoresis in 2% agarose and staining with ethidium bromide (29).

Vectorette PCR was performed as described previously (23) by using primer IS1356-A (Table 1) as the target primer. Vectorette libraries were constructed with NAs from strain LCDC 92-498 (ET12, RT20) for *Bam*HI, *Bcl*II, *Bgl*III, *Eco*RI, *Hind*III, *Nhe*I, *Sal*I, *Spe*I, and *Xba*I. This isolate is a member of the group of strains implicated in the spread of *B. cepacia* ET12 between the United Kingdom and Canada (10, 13, 31). Amplification was performed as described above by using a two-step thermocycling profile of 30 cycles of 94°C for 30 s and 72°C for 3 min. The reaction mixtures were analyzed on a 1% low-melting-point agarose gel, and the resulting amplicons were excised from the gel and purified with the Wizard PCR Prep Purification system (Promega, Madison, Wis.).

Cloning and sequencing of IS402-IS1356. A bacteriophage library was constructed from strain LCDC 92-498 by using the ZAP Express Cloning Kit (Stratagene, La Jolla, Calif.). The probe was prepared by amplifying NA from strain LCDC 92-498 with the primers IS1356-A and IS1356-B (Table 1) in the presence of digoxigenin-11-uridine-5'-triphosphate (DIG). PCR conditions were identical to those used to detect the IS; however, the deoxynucleoside triphosphates were substituted with DIG Labeling Mix (Boehringer Mannheim). Positive clones were identified with the DIG DNA Detection Kit (Boehringer Mannheim) according to the manufacturer's directions. After purification of the bacteriophage clones, the phagemids were excised as directed in the ZAP Express Kit and plasmid DNA was purified with the QuiaWell Plus Plasmid purification system (Quiagen, Chatsworth, Calif.) as recommended by the manufacturer.

Sequencing was performed on an ABI 373 automated DNA sequencer by using the Prism Dye Terminator sequencing kit (Applied Biosystems). Sequencing primers were designed on the basis of the acquired data as required to complete the sequence. Sequence analysis was performed with the various programs supplied with PG/Gen (Intelligenetics, Mountain View, Calif.) and Lasergene (DNASar, Madison, Wis.). Phylogenetic analysis was performed with PAUP, version 3.0 (37).

IS designation and nucleotide sequence accession number. The IS1356 designation was from Esther M. Lederberg (Stanford University School of Medicine, Stanford, Calif.), under the auspices of the Plasmid Reference Centre Prefix Registry.

The IS402-IS1356 sequence has been assigned GenBank accession number U44828.

RESULTS

Frequency of targeted IS elements in *B. cepacia*. The frequencies of occurrence of targeted IS elements in *B. cepacia* isolates from environmental, nosocomial, and CF patient sources are recorded in Table 2. Overall, IS402 was found in 68.7% of the isolates, IS406 was found in 22.2% of the isolates, IS407 was found in 48.5% of the isolates, IS408 was found in 53.5% of the isolates, and IS1356 was found in 71.7% of the isolates. In addition to these IS elements, primer sets were also designed to amplify IS401 (2), but no amplicons were detected in this collection of isolates (data not shown). There were no apparent linkages between the presence of these IS elements and an ET or an RT, with one notable exception. The primers targeting IS402, in addition to detecting the IS element, also primed an additional amplicon of approximately 650 bp in some isolates. This anomaly was restricted to strains designated ET12, which is the ET of isolates documented to be highly transmissible in CF patients (10, 13, 31). Sequence data revealed that the 650-bp amplicon consisted initially of the

TABLE 2. Distribution of IS elements in *B. cepacia* isolates

Source	No. of isolates	No. (%) of isolates with the indicated IS element					
		IS402	IS406	IS407	IS408	IS1356	IS402-IS1356
Environmental	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	0 (0.0)
Nosocomial	15	10 (66.7)	7 (46.7)	2 (13.3)	12 (80.0)	4 (26.7)	0 (0.0)
CF patients	82	58 (70.7)	15 (18.3)	46 (56.1)	41 (50.0)	66 (80.5)	36 (43.9)
Total	99	68 (68.7)	22 (22.2)	48 (48.5)	53 (53.5)	71 (71.7)	36 (36.4)

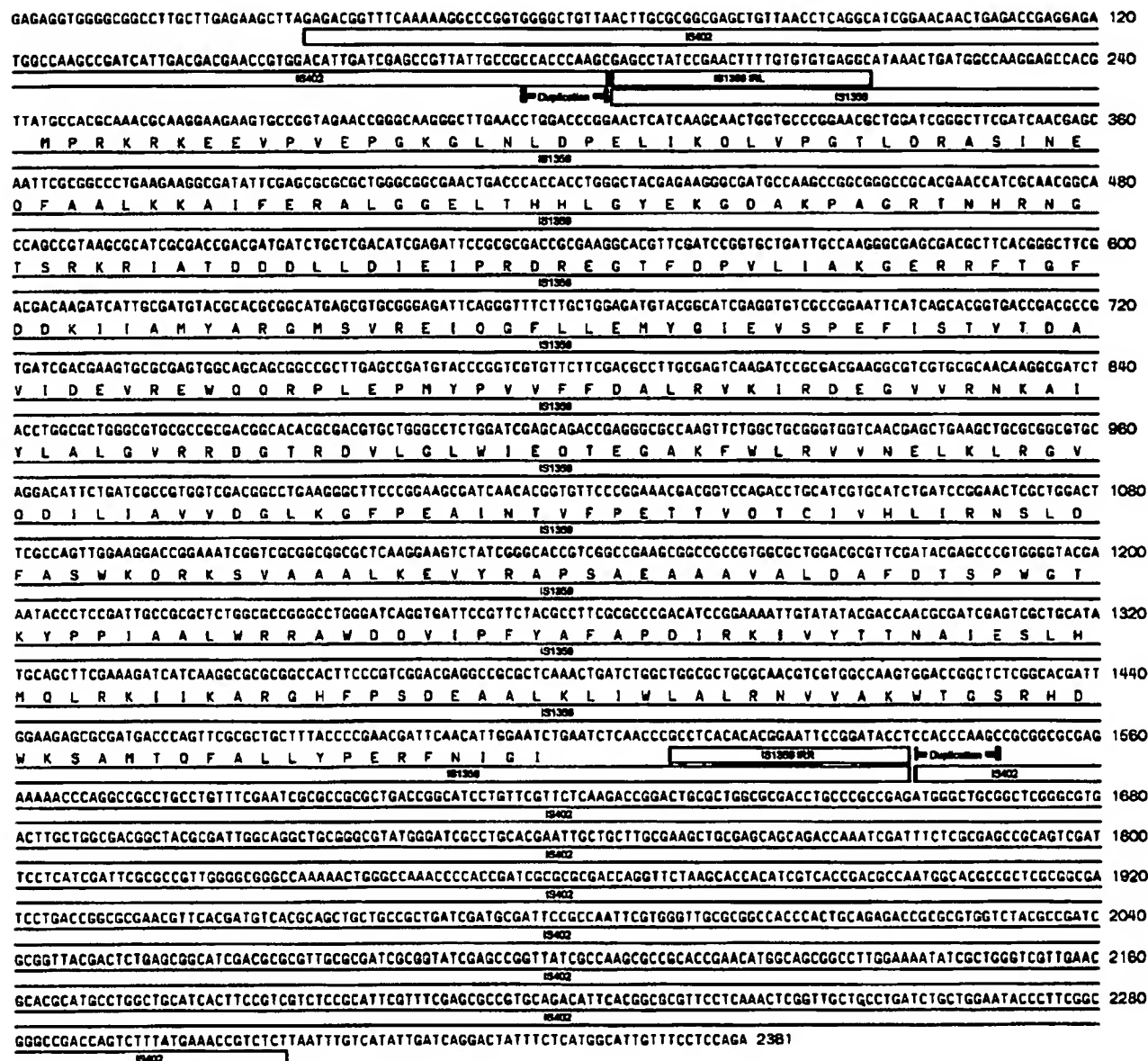


FIG. 1. Nucleotide sequence of IS402-IS1356 and the amino acid sequence of the putative transposase of IS1356. The pertinent features of the IS402-IS1356 element are illustrated below the nucleotide sequence. The locations of IS402 and IS1356 and their terminal inverted repeats are shown by the open boxes. The 10-bp duplication of IS402 due to the insertion of IS1356 is indicated by the solid bars.

IS402 sequence, but this was interrupted after 154 bp and was succeeded by the sequence of IS1356.

Cloning and characterization of IS402-IS1356. Through the use of the vectorette PCR, an amplicon of approximately 1,300 bp was obtained from the *Bcl*II library and was used for sequencing. The *Bgl*II and *Eco*RI libraries also produced amplicons, but these were considerably smaller in size and were not investigated further. After sequencing of the amplicon, further attempts at "gene walking" through the use of vectorette PCR proved unsuccessful because of the large number of unresolvable amplicons obtained. From the bacteriophage library 10 candidate clones were selected for sequencing and allowed for the identification of three different insertion sites. Insertion into IS402 is shown in Fig. 1. The two other sites identified

were 5'-CTGACCGGCGG-IS1356-CCACCGGTGA-3' and 5'-CGTTGTCTCG-IS1356-3'. The clone containing the latter insertion site did not contain the full IS1356 sequence, and therefore, the 3' insertion sequence is not known.

The IS402-IS1356 element consists of the IS402 reported by Ferrante and Lessie (6), including the 3-base duplication (5'-TTA-3') at the insertion site. Although certain sequence differences were detected between the IS402 sequence reported previously and the one present in the hybrid, these differences were not considered significant. The IS402 sequence is interrupted after 154 bases by IS1356, resulting in a 10-bp duplication at the insertion site. IS1356 is 1,353 bp in length and terminates at either end by imperfect inverted repeats. The left-hand inverted repeat (IRL) is 27 bp in length and the

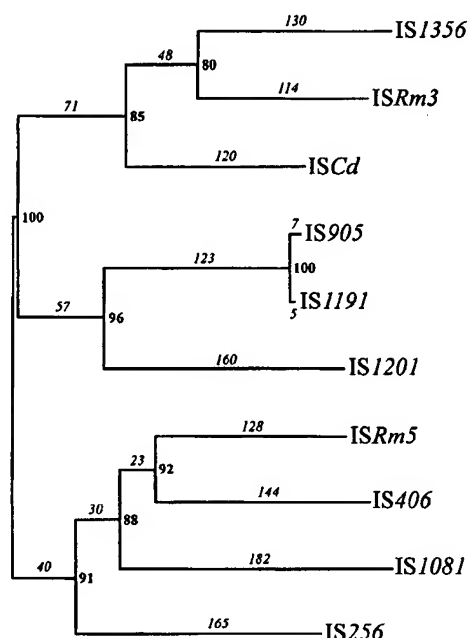


FIG. 2. Phylogenetic relationships among the putative transposases of various IS elements and that of IS1356. The dendrogram was generated after 100 bootstrap replications of branch-and-bound searches by using PAUP, version 3.0. The numbers along the branches indicate the branch lengths, and the numbers at the branch nodes are the respective bootstrap values. The transposases compared with that of IS1356 were those found on ISRm3 from *R. meliloti* (40), ISCd from *Corynebacterium diphtheriae* (22) (ISCd is an unofficial designation for this element), IS905 from *Lactococcus lactis* (5), IS1191 from *Streptococcus thermophilus* (11), IS1201 from *Lactobacillus helveticus* (39), ISRm5 from *R. meliloti* (14), IS406 from *B. cepacia* (41), IS1081 from *Mycobacterium bovis* (4), and IS256 from *Staphylococcus aureus* (3).

right-hand inverted repeat (IRR) is 29 bp in length, with seven mismatches over the common region. IS1356 contains one major open reading frame of 1,260 bp which codes for a putative transposase. This transposase showed significant homologies to several others found in the Swiss-Prot 31 database, with the most significant homologies of 59% to ISRm3 found in *Rhizobium meliloti* (40) and 49% to an undesignated IS found in *Corynebacterium diphtheriae* (22). The phylogenetic relationships between several of the more closely related transposases are shown in Fig. 2.

In order to determine if the IS402-IS1356 element had a conserved insertion site, primers which would amplify either the 5' or 3' insertion sites were designed, and the resulting amplicons were sequenced (data not shown). All isolates in which the IS402-IS1356 hybrid IS element was detected yielded an amplicon of the predicted size with a sequence identical to that originally identified.

Distribution of targeted IS elements and *cblA* pilin subunit genes among several *B. cepacia* ETs. Table 3 summarizes the distributions of the IS elements and the *cblA* pilin subunit genes in our collection of 99 strains representative of 20 ETs. When IS1356 was detected in strains, the primers did not permit discrimination between IS elements found as the hybrid and IS1356 located at other sites. The initial identification of the IS402-IS1356 element was accomplished by observing the 650-bp band obtained with the IS402 primer set; however, in these strains successful amplification of the target was difficult to reproduce, so the presence of the hybrid was confirmed by screening all isolates with the IS402-A and IS1356-B primers to obtain a 592-bp amplicon (data not shown).

Two of the ET12 isolates studied were found to lack the IS402-IS1356 element. One of these isolates was found to possess all of the other IS elements investigated except IS406, and the other possessed IS408 and IS1356. Neither of these two ET12 isolates lacking the hybrid element were clearly associated with the epidemic, in that one is a reference strain used many years ago to establish the serotyping scheme for *B. cepacia* (18) and the other came from a CF patient resident in

TABLE 3. Distribution of targeted genes in *B. cepacia* strains representing 20 ETs

ET	No. of isolates tested	Source ^a	No. of isolates with the indicated IS or gene						
			IS402	IS406	IS407	IS408	IS1356	IS402-IS1356	<i>cblA</i>
1	1	CF	1	0	1	1	1	0	0
2	1	CF	1	0	1	0	0	0	0
3	1	NS	1	0	0	1	0	0	0
4	2	CF	0	0	0	0	1	0	0
5	1	CF	0	0	1	0	0	0	0
6	3	CF	0	0	0	0	2	0	0
7	1	CF	0	0	0	0	0	0	0
8	2	NS	2	1	2	1	0	0	0
9	1	ENV	0	0	0	0	0	0	0
10	1	NS	0	0	0	1	0	0	0
11	5	NS	1	3	0	4	2	0	0
12 ^b	38	CF	37	10	37	32	38	36	38
13 ^c	1	CF	1	0	1	0	1	0	1
14	1	CF	0	0	1	0	1	0	0
15	1	NS	1	0	0	1	1	0	0
16	18	CF	9	2	2	1	18	0	0
17	12	CF	10	2	2	7	5	0	0
18	4	NS	4	4	0	4	0	0	0
19	1	ENV	0	0	0	0	1	0	0
20	4	CF	0	0	0	0	0	0	0

^a CF, CF patient; ENV, environmental; NS, nosocomial.

^b Epidemic transatlantic clone (Canada and the United Kingdom).

^c Isolate from a Canadian CF patient in a province remote from Ontario and with no known epidemic association.

a remote area of northern Ontario with no known association with areas of the provincial epidemic in the south. The *cblA* pilin subunit gene was found in all ET12 isolates tested and one strain of ET13 from an adult CF patient in a province remote from Ontario. There is no documented evidence of an epidemic association or the spread of this single ET13 isolate, which differs in only one esterase allele from ET12 and which is IS402-IS1356 negative (Table 3). The *cblA* amplicon from this isolate was subjected to sequence analysis and was found to be identical to that in the ET12 isolates (data not shown).

DISCUSSION

In our earlier study (13), we demonstrated that while RTs appear to be highly variable in a geographical context, ETs seem relatively stable in a population of *B. cepacia* isolates from particular clinical sources and may, in fact, be the best indicator of a clonal distribution. Considering the large number of ISs known to reside in *B. cepacia* strains and their propensity to cause genetic rearrangements, the variability of RTs is not surprising. Since a number of IS elements found in *B. cepacia* isolates have been shown to affect the expression of associated genes in other circumstances (6, 16, 30, 41), we questioned if there were any linkages between carriage of particular IS elements, transmissibility, and/or virulence and ET type.

During the course of our investigations we succeeded in identifying a previously unreported IS element, which has been designated IS1356. This sequence has a structure typical of ISs in that it is terminated by inverted repeats, contains an open reading frame which spans virtually its entire length, and codes for a putative transposase. An interesting feature of IS1356 is the high degree of similarity of the transposase to a variety of others identified on IS elements from a wide distribution of organisms. During the characterization of IS*Rm5*, Laberge et al. (14) observed that there appears to be a family of IS elements, of which IS1356 is now a member, which share a common ancestry, even though the hosts of these IS elements are from highly divergent bacterial families. From an evolutionary standpoint these similarities are quite intriguing. Similarities between IS elements from *B. cepacia* and *R. meliloti* strains could possibly be explained by their close association with respect to environmental habitat, but the ancestral relationship between these organisms and the other members of this family is difficult to fathom. Another interesting feature of IS1356 is its insertion into IS402. While this arrangement is not unprecedented (14, 32), it proved to be unique to a particular clonal cluster of *B. cepacia* (the ET12 cluster) and was not found in other isolates. In addition, the site of insertion of the IS402-IS1356 element was identical in all of the isolates investigated, further supporting the clonal nature of this group of isolates.

Although a large number of IS elements have been identified in *B. cepacia* strains, our study of their distribution was limited by the fact that only a small number of these have been sequenced. For those for which sequence data were available, no relationship between ET, RT, and IS carriage was found with the exception of the association of the IS402-IS1356 element with ET12. The IS402-IS1356 element was only found in *B. cepacia* strains isolated from CF patients and was restricted to ET12, with 36 of 38 (94.7%) of these isolates harboring the hybrid. Two of the 38 ET12 isolates examined lacked the IS402-IS1356 element, although they both contained IS1356 alone. Neither strain could be directly linked to the epidemic clone, because one was an early isolate used to establish a serotyping scheme and therefore was temporally unrelated and the other was isolated from a CF patient living in a remote

location in Ontario with no obvious connections to the urban epidemic in that province.

The current study did not directly address this issue; however, it was noted that there appeared to be a certain bias in the distribution of IS elements among the clinical isolates with respect to the source of the host from which the strains were isolated. The IS elements IS406 and IS408 appeared more frequently in nosocomial isolates, whereas the IS elements IS407 and IS1356 appeared more frequently in isolates from CF patients. IS402 was equally distributed between the two groups, and the IS402-IS1356 element was found exclusively in CF patients and was restricted to ET12 isolates. Admittedly, the number of nosocomial isolates investigated was low and a wider sampling may alter the apparent bias in IS element distribution; however, it is also possible that this bias may reflect an ancestral branching of *B. cepacia* strains with respect to their clinical significance and association with a particular disease manifestation.

At present it is not known if the IS402-IS1356 element is linked to the apparent increase in transmissibility and virulence of *B. cepacia* ET12, only that there is an "association." It is quite possible that the hybrid IS is simply a marker for this particular lineage of *B. cepacia*. Although the present study was not designed to demonstrate a direct effect of IS elements on virulence, the possibility of this type of association is not precluded. The importance of IS elements in the adaptability of *B. cepacia* isolates should not be ignored, and while they may not be directly related to virulence, they may, nonetheless, play an important indirect role in pathogenicity by assisting in the acquisition of virulence factors in certain strains.

The pathogenicity of *B. cepacia* and the implications of colonization with this organism are of considerable interest to those involved in the management of CF patients. Anecdotal evidence has recently linked a highly-transmissible ET12 clone currently endemic in Ontario, Canada, to that causing an epidemic in the United Kingdom (10, 13). Sun et al. (36) demonstrated that the infectious, transatlantic clone was characterized by both *cblA* gene sequences and a similar chromosomal fingerprint. In the current study we found that the *cblA* pilin subunit gene was present in 100% (38 isolates from 18 patients) of the epidemic ET12 isolates in our collection of *B. cepacia* strains. However, it was also found in one ET13 isolate from an adult CF patient living in a province which has never been particularly associated with epidemics of any nature. Since Sun et al. (36) also identified a single unrelated isolate with a divergent *cblA* NA sequence, the PCR amplicon from our ET13 isolate was sequenced and was found to be identical to that characterized for the epidemic strain. In view of this evidence it would appear that, upon a wider sampling, the *cblA* pilin subunit gene may prove to be more invariant among unrelated strains than reported by Sun et al. (36).

In addition to the *cblA* pilin subunit gene association, Sun et al. (36) also presented limited ribotyping data and phylogenetic analysis which appeared to support the clonal nature of these strains. In their investigation, two very similar ribotype patterns were identified for the epidemic strains, and it was suggested that this general profile could also be used to identify these strains. However, in our earlier work (13), in addition to the two RT patterns identified by Sun et al. (36), we identified five other RT patterns within the epidemic strains using an identical methodology. It should also be noted that the group of bands which were invariant among the ET12 isolates were also seen in other unrelated isolates of various ETs. With this in mind it would appear that the claims made by Sun et al. (36) as to the usefulness of ribotyping as a screening method were somewhat premature and perhaps overly optimistic.

In view of the potential impact on future protocols for the management of *B. cepacia*-colonized CF patients, caution is advised in the screening of isolates solely on the basis of markers attributed to a single highly transmissible strain. It is clear that the *cblA* pilin subunit gene, the ET12 genotype, and the IS402-IS1356 element appear to correlate well with this one particular epidemic strain, and the ability to identify this clone is not without value. However, as Sun et al. (36) also acknowledge, other distinct and highly transmissible epidemic lineages for which no genetic marker is currently available appear to exist. If any of these methods were prematurely implemented to screen CF patients, we would risk placing patients in the wrong groups, and the consequences in the case of these CF patients would be clinically and personally tragic.

The genome of *B. cepacia* is exceedingly complex, having three large chromosomal elements and a large plasmid, an arrangement apparently unique among eubacteria (24), and more genes involved in metabolism and pathogenicity need to be identified in order to provide important information on the virulence of clinical isolates. In view of the current data, it would appear that several genetic loci may need to be characterized and to agree in order to accurately assign clinical isolates to epidemic lineages. Until more is known about the pathogenicity of *B. cepacia* and the resulting implications to the CF community, we recommend that caution be applied before establishing any definitive phenotypic or genotypic screening criteria.

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Genotypic and phenotypic relationships in *Burkholderia cepacia* isolated from cystic fibrosis patients and the environment

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P. WIGLEY AND N.F. BURTON. 1999. Twenty-one strains of *Burkholderia cepacia* isolated from the environment, and 21 clinical strains isolated principally from sputum of cystic fibrosis (CF) patients, were characterized genotypically by macrorestriction analysis (genome fingerprinting) and PCR ribotyping, and phenotypically by susceptibility to antibiotics and the ability to macerate onion tissue. The plasmid content of the strains was also investigated. Environmental isolates showed a high degree of genetic variability, all strains differing from both one another and the CF isolates. The CF isolates were less variable, with common strains found in patients attending three geographically distinct CF centres. Phenotypic variation was found both within and between CF and environmental strains. Generally, CF isolates displayed higher levels of antibiotic resistance, while the ability to macerate onion tissue was more prevalent amongst environmental isolates. Plasmids were more frequently found in CF isolates, but were of similar size in both groups of strains. Such variability is not surprising in view of the existence of multiple genomovars within the *B. cepacia* complex.

INTRODUCTION

The Gram-negative bacterium *Burkholderia cepacia* was originally described as a phytopathogen of onion (Burkholder 1950) and subsequently as a saprophyte in soils and waters (Morris and Roberts 1959). More recently, it has emerged as an opportunistic pathogen of man, particularly among cystic fibrosis (CF) and chronic granulomatous disease patients (Govan *et al.* 1996), as a nosocomially acquired infection in a hospital intensive care unit (Pegues *et al.* 1996), and amongst oncology patients (Pegues *et al.* 1993). *Burkholderia cepacia* displays intrinsic resistance to a range of antibiotics (Wilkinson and Pitt 1995), shows diverse degradative properties including the degradation of recalcitrant polychlorinated aromatic compounds, and produces a number of compounds antagonistic to phytopathogenic fungi. Such properties have led to interest in *B. cepacia* as a possible agent of bioremediation or biocontrol (Govan *et al.* 1996).

Characterization of *B. cepacia* infecting CF patients in epidemiological studies in the UK (Govan *et al.* 1996; Pitt *et al.* 1996) and the USA (Johnson *et al.* 1994) has shown strong evidence of an epidemic strain of *B. cepacia*, and of person-to-person transmission amongst CF patients. The risk posed to CF patients from environmental reservoirs of *B. cepacia* strains and the relationship between clinical and environmental isolates is unclear, with contradictory evidence for phenotypic differences between isolates from CF patients and the environment (Govan *et al.* 1996). Isolates from CF patients and nosocomially acquired infections have been shown to be incapable of causing the maceration of onion tissue *in vitro* whereas environmental isolates have been shown to macerate onion tissue and display high levels of pectinolytic activity (Gonzalez and Vidaver 1979; Bevivino *et al.* 1994). However, a number of other CF isolates, including the epidemic strain, have been shown to cause maceration of onion by other workers (Butler *et al.* 1995). The inability of environmental isolates to adhere to human uroepithelial cells has been suggested as evidence that environmental *B. cepacia* is incapable of causing infection in man (Bevivino *et al.* 1994), though more recent evidence has suggested that the environment may be a potential source of infection to CF

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patients (Cazzola *et al.* 1996). Such contradictory evidence has led to caution being advised in the use of *B. cepacia* as an agent of bioremediation or biocontrol until the relationship between clinical and environmental isolates of *B. cepacia* has been fully assessed (Butler *et al.* 1995; Govan *et al.* 1996).

Phenotypic differences between *B. cepacia* strains such as those described above, along with genetic variability in both environmental and clinical isolates (Pitt *et al.* 1996; Wise *et al.* 1996), have led to suggestions that the phylogeny of the species should be revised (Govan *et al.* 1996; Vandamme *et al.* 1997). Yohalem and Lorbeer (1994) placed *B. cepacia* isolates into four groups or phenoms on the basis of a range of phenotypic properties. Genomic fingerprinting by pulsed field gel electrophoresis (PFGE) and arbitrarily primed PCR (AP-PCR) found four distinct genetic groups or genomovars amongst clinical and environmental isolates of *B. cepacia* (Vandamme 1995; Revets *et al.* 1996). Genomovars have been defined as genetically different groups of bacteria that are indistinguishable by current phenotypically based typing methods (Ursing *et al.* 1995). Recent evidence has suggested that *B. cepacia* is formed by a complex of five genomic species: *B. cepacia* genomovars I, II and IV, *B. vietnamensis* and the newly proposed species, *B. multivorans* (Vandamme *et al.* 1997).

Although strains of *B. cepacia* infecting CF patients in the UK and North America have been well characterized phenotypically and by molecular typing methods (Pitt *et al.* 1996; Henry *et al.* 1997), there has been little characterization of environmental isolates found in the UK. Butler *et al.* (1995) investigated the phenotypic properties of 12 environmental isolates of *B. cepacia* and undertook genomic analysis by macrorestriction digests followed by PFGE. These isolates were found to differ both genetically and phenotypically from each other and from isolates infecting CF patients. However, most of these isolates were obtained from tropical houses in botanical gardens. These isolates could be described as coming from an unusual habitat in terms of the environmental population of *B. cepacia* that occurs in the UK, and CF patients are much less likely to come into contact with such isolates than with those occurring in the natural rural and urban environment of the UK. In this study, isolates obtained from the environment (soils, rhizosphere and waters) from both urban and rural areas were characterized phenotypically by their ability to macerate onion tissue *in vitro* (Lelliott and Stead 1987) and their susceptibility to a range of antibiotics, genetically through molecular typing by PCR ribotyping, PFGE macrorestriction analysis, and through their plasmid content and size. These isolates were compared with *B. cepacia* strains isolated from CF patients living in the South Wales area, and with isolates from other European CF centres, to investigate relationships and variations between *B. cepacia* isolates from CF patients and those in the environment to which the patients may be exposed.

MATERIALS AND METHODS

Bacterial strains

Twenty-one environmental strains were isolated from soils, waters and rhizosphere at 106 sites in South Wales. Sites were chosen from urban, rural and industrial areas to give a full representation of environmental *B. cepacia* in South Wales. For soil samples, 2–3 g soil were collected into a sterile universal container, 10 ml sterile water were added and the soil suspended by vortex mixing for 5 min. The sample was allowed to settle before 100 µl of the aqueous layer was removed and plated directly onto each of the selective media used. For the rhizosphere samples, plant roots were gently removed from soil and treated as for the soil samples. For water samples, around 10 ml water and sediment were collected from streams and rivers in an upstream direction, or from the edges of lakes and reservoirs. Samples were vortex mixed for 5 min. After mixing, 100 µl of the sample were plated directly onto each of the selective media.

Each sample was plated onto a range of selective media: *B. cepacia* selective agar (Mast Diagnostics, Bootle, UK); TB-T agar (Hagedoorn *et al.* 1987); TB-T agar supplemented with 5 g ml⁻¹ gentamicin; and a minimal agar medium containing 20% onion homogenate and tetracycline at 20 g ml⁻¹ with and without the addition of gentamicin at 5 g ml⁻¹ (Wigley 1998). The samples were incubated at 30 °C for 48 h. Colonies found growing were Gram stained and all Gram-negative rods were sub-cultured onto fresh media prior to identification, principally by the API 20 NE system (BioMérieux, Marcy L'Etoile, France). Twenty-one isolates were identified as *B. cepacia*, a recovery rate of 20% from samples tested. The use of a range of selective media was found to increase the recovery rate of *B. cepacia* by around 70% compared with the use of the commercial *B. cepacia* medium alone, suggesting an underestimation of *B. cepacia* in the environment in some previous studies (Fisher *et al.* 1993; Mortensen *et al.* 1995).

Twenty-one isolates from the sputum of CF patients attending the Cardiff CF centre were kindly provided by Mr Alan Paull of the Public Health Laboratory Service, Cardiff. A number of isolates from CF centres in Strasbourg and Scotland were kindly provided from the collection of Prof. J.R.W. Govan, University of Edinburgh. The species type strain NCPPB 2993 (ATCC 25416) was obtained from the National Collection of Plant Pathogenic Bacteria (Harpden, Hertfordshire, UK). Stock isolates were maintained at –70 °C in nutrient broth with 20% glycerol as a cryoprotectant.

In vitro maceration of onion tissue

In vitro maceration of onion tissue was investigated by a modified version of the method of Lelliott and Stead (1987).

Clean, sound, disease-free onions (*Allium cepa*) were wiped with 90% v/v alcohol before cutting aseptically into slices of approximately 5 mm in thickness. Onion slices were placed into sterile Petri dishes and nicks of approximately 2 mm made in the tissue surface. Overnight nutrient broth cultures (100 µl) of each *B. cepacia* isolate to be tested (approximately 10^7 cfu ml⁻¹) were inoculated onto the surface of the onion tissue, with sterile saline and *Pseudomonas aeruginosa* isolates as controls. Onion slices were incubated at 30 °C for 48 h, after which onion maceration and discoloration were assessed with the aid of a sterile needle as a probe.

Antibiotic susceptibility

Antibiotic resistance profiles were determined by the modified Kirby Bauer Disc Diffusion test using the WHO recommended method (Vandepitte *et al.* 1991). The following discs used were selected on the basis of those recommended by Acar and Goldstein (1985) and the WHO (Vandepitte *et al.* 1991) for *Pseudomonas* species: 25 µg amoxicillin, 30 µg ceftazidime, 15 µg erythromycin, 10 µg gentamicin, 5 µg novobiocin, 10 IU penicillin G, 25 µg sulphamethoxazole and 30 µg tetracycline (Oxoid). Plates were incubated at 35 °C for 24–48 h. *Escherichia coli* NCTC 10418 was included as a control for interpretation of results with each batch of *B. cepacia* isolates tested.

Minimum inhibitory concentration (MIC) was determined for piperacillin, ceftazidime, gentamicin and sulphamethoxazole by an agar dilution method using doubling dilutions of antibiotics ranging from 512 µg ml⁻¹–1 µg ml⁻¹. Overnight cultures of *B. cepacia* isolates (approximately 10^7 cfu ml⁻¹) were inoculated onto the surface of the plates using a Mast multipoint inoculator (Mast Diagnostics). Inoculated plates were incubated at 35 °C for 24–48 h. Minimal inhibitory concentration was taken as the lowest concentration that inhibited growth of the isolate. *Escherichia coli* NCTC 10418 was included as a control.

Isolation and sizing of plasmid DNA

Isolation and size estimation of plasmid DNA was according to the methods of Rochelle *et al.* (1985) based on Kado and Liu (1981). The methods were chosen for their accuracy in determining the sizes of small and large plasmids, with minimal shearing of large plasmids and without the need for restriction digests of the plasmid DNA. Overnight bacterial culture in nutrient broth (1.5 ml) was pelleted at 13 000 g for 4 min, the supernatant fluid discarded and the pelleted cells mixed with lysing solution (0.15 g Tris, 1.75 g sodium dodecyl sulphate, 425 µl sodium hydroxide 2 mol l⁻¹, in 25 ml deionized water, pH 12.6) and incubated at 65 °C for 90 min. Phenol/chloroform (150 µl) (Sambrook *et al.* 1989) was added and an emulsion formed by vigorous shaking. The emulsion

was broken by centrifuging at 13 000 g for 4 min. A 75 µl aliquot of the upper aqueous layer containing the plasmid DNA was removed gently with a wide bore pipette tip to a fresh microcentrifuge tube; 20 µl of this sample was loaded into a 0.7% agarose gel in TBE buffer in a Gibco BRL Horizon 58 gel electrophoresis tank (Life Technologies) and run at 4 V cm⁻¹ for 90 min. The gel was stained in 0.5 g ml⁻¹ ethidium bromide and destained under running water before photographing under u.v. transillumination.

Plasmid sizes were estimated by comparison with mobility of plasmids of known sizes, after regression analysis of the plot of log₁₀ molecular size (in kbp) against log₁₀ mobility (distance travelled from origin in mm). The *E. coli* strains V517 (Macrina *et al.* 1978) containing eight plasmids of 55 to 2 kbp, and 39R (Threlfall *et al.* 1986) containing four plasmids of 151 to 7 kbp, were used as size markers.

PCR ribotyping

PCR ribotyping was conducted by the method of Ryley *et al.* (1995) using primers from a conserved spacer region between the 16S and 23S Gram-negative bacterial genes. PCR products were analysed by agarose gel electrophoresis on a high resolution gel containing 'Infinity' agarose extender (Applied Gene Oncor, Durham, UK) to give a gel approximately equivalent to 3% agarose. This method was previously used to type *B. cepacia* isolates from CF patients in Denmark (Ryley *et al.* 1996), and gives more rapid results than with *TaqI* digestion of PCR products followed by electrophoresis. *Taq* DNA polymerase (at 5000 units ml⁻¹) was supplied by Applied Gene Oncor. Primers were supplied by MWG Biotech (Milton Keynes, UK). Primer 1 (C1): 5'-TTG TAC ACA CCG CCG GTC A-3' was supplied at 39.3 pmol ml⁻¹. Primer 2 (C2): 5'-GCT ACC TTA GAT GTT TCA GTT C-3' was supplied at 54.1 pmol ml⁻¹. For each set of reactions, a control was set up using distilled water in place of template DNA. PCR markers (Sigma) were included in each gel.

Macrorestriction analysis

The macrorestriction analysis (genomic fingerprinting) method used was developed from that of Grouthens *et al.* (1988) for the analysis of *Ps. aeruginosa*. *Burkholderia cepacia* was harvested from overnight cultures on nutrient agar using 5 ml SE buffer (0.075 mol l⁻¹ NaCl, 0.025 mol l⁻¹ EDTA). The cell suspension was drawn off with a pipette and turbidity adjusted to MacFarland standard three (approximately equivalent to 9×10^8 cells ml⁻¹) with SE buffer; 500 µl of this suspension was mixed with 500 µl of 2% molten low gelling temperature agarose (Sigma Chemical Co.) maintained at 40 °C, pipetted into disposable plug moulds (BioRad) and allowed to set. Plugs were removed and twice treated for 24 h with lysis buffer (N-lauroyl sarcosinate 1%, 0.5 mol l⁻¹

EDTA, 500 µg ml⁻¹ proteinase K, pH 9.5) at 56 °C. Plugs were then washed four times in TE buffer (Sambrook *et al.* 1989). The resulting agarose-embedded genomic DNA could be stored at 4 °C for up to 2 weeks prior to use.

The embedded DNA was digested with restriction enzymes *Xba* I and *Not* I (Promega) using 50 units of enzyme per plug in 200 µl of the appropriate digestion buffer (BioRad Laboratories 1992) for 1 h at 37 °C (Sambrook *et al.* 1989).

Digested plugs were sealed in the wells of a 1% pulsed field agarose gel in 0.5 TBE buffer (Sigma) in a CHEF DR II apparatus (BioRad), and run for 21 h at 5 V cm⁻¹ with initial pulse times of 5 s and final pulse times of 100 s in 0.5 TBE buffer at a temperature of 8 °C. Size markers of 225–2200 kbp and/or 0.1–200 kbp (Sigma), or *Saccharomyces cerevisiae* size markers (BioRad), were included on each gel. Gels were stained in ethidium bromide and destained in running water, then photographed under u.v. transillumination.

RESULTS

In vitro maceration of onion

The degree of maceration of onion tissue was assessed as follows:

- 0 No maceration, discoloration or odour. Typified by sterile saline control.
- 1 No maceration, slight discoloration and 'sour' odour.
- 2 Patches of maceration and discoloration, 'sour' odour.
- 3 Maceration in majority of tissue. Yellow to yellow-brown discoloration. Strong 'sour' odour.
- 4 Severe maceration throughout tissue. Yellow-brown discoloration and very strong 'sour' odour. Typified by NCPPB 2993 (type strain and pathotype).

The levels of pathology caused by each isolate are shown in Table 1.

Plasmid size and content

One or more plasmids were detected in 16 out of 21 *B. cepacia* CF isolates (76%), and seven out of 21 environmental isolates (33%). Plasmids detected and their sizes are shown in Table 1.

Antibiotic susceptibility

All *B. cepacia* isolates from CF patients and the environment were found to be resistant to penicillin G, amoxicillin, erythromycin and novobiocin; 76% of environmental isolates (16 of 21) and 90% of CF isolates (19 of 21) were also found to be resistant to tetracycline. Resistance levels to piperacillin, gentamicin, ceftazidime and sulphamethoxazole varied more

between the environmental and CF isolates and are shown in Table 2. The MIC values for these antibiotics were generally higher amongst the CF isolates than amongst environmental isolates.

PCR ribotyping

Different patterns of PCR products were found for all 21 environmental isolates, with between two and eight bands ranging in size from 700 to 2500 bp occurring. This indicated 21 ribotypes that differed from all the CF isolates tested and also the species type strain (Fig. 1 illustrates the range of results). All the CF isolates from the Cardiff centre, with the exception of C93, gave an identical or near identical pattern with a band at around 800 bp and two other bands at around 2000 and 2500 bp. The same pattern of products was found to occur with *B. cepacia* isolates from the Strasbourg (J543) and Dundee (C1858) CF centres. The other isolates from these centres differed in pattern from the Cardiff CF isolates, all the environmental isolates and the type strain NCPPB 2993 (ACTC 25416).

Macrorestriction analysis

Following digestion with *Xba* I, around 40 fragments of 1–200 kbp were found by PFGE. Each of the 21 environmental *B. cepacia* isolates produced a unique restriction pattern that differed from all the patterns obtained with isolates from CF patients (Fig. 2). All Cardiff CF *B. cepacia* isolates, again with the exception of C93, produced patterns that were identical or near identical. The Strasbourg CF isolate J543 and the Scottish CF isolate C1858 also produced this digest pattern. Digestion with *Not* I produced the same similarities and differences between isolates.

DISCUSSION

All environmental isolates tested in this study were found to differ genetically from each other, indicating that there is great genetic variability in the environmental population of *B. cepacia* in South Wales. High levels of variability have previously been described in environmental populations of *B. cepacia* (Wise *et al.* 1996). Sixty-five unique electrophoretotypes (ETs) were found by multilocus enzyme electrophoresis in 217 *B. cepacia* isolates collected from a 5 km stretch of stream over a period of 32 d. It is also notable that genetic differences were found in isolates obtained from sites close together. Examples of this are PW1, PW2 and PW6, isolated over a 100 m stretch of stream, and PW5 and PW7, isolated from soil samples taken a few metres apart. The 12 environmental isolates from botanical gardens described by Butler *et al.* (1995) gave 11 different profiles by macrorestriction analysis, though it was notable that isolates from the same source

Table 1 Plasmid content and sizes and onion macerating properties of *Burkholderia cepacia* isolates of clinical and environmental origin

Isolate	Source	Plasmid content and size (kbp)	Onion maceration (0-4 scale)
NCPB 2993	Rotten onion	224	4
	Species type strain		
C1	CF patient, Cardiff	134	1
C5	CF patient, Cardiff	134	1
C11	CF patient, Cardiff	134	0
C23	CF patient, Cardiff	139, 24, 13	1
C49	CF patient, Cardiff	2.1, 1.7	0
C51	CF patient, Cardiff	ND*	0
C59	CF patient, Cardiff	20	0
C79	CF patient, Cardiff	90	1
C81	CF patient, Cardiff	40	0
C93	CF patient, Cardiff	ND	1
C96	CF patient, Cardiff	109, 2.1, 1.7	0
C116	CF patient, Cardiff	144, 29.3, 2.1, 1.7	1
C187	CF patient, Cardiff	51, 4.4, 4.0	0
C190	CF patient, Cardiff	ND*	1
C205	CF patient, Cardiff	ND*	0
J543	Clinical, Strasbourg	90	1
J478	Clinical, Strasbourg	152	1
A548	CF patient, Edinburgh	ND*	2
A562	CF patient, Edinburgh	70, 4.5	3
C1858	CF patient, Dundee	ND*	1
C1860	CF patient, Aberdeen	185, 3.4	0
PW1	Stream water	ND*	1
PW2	Stream water	132, 51, 31, 16, 9, 8, 3.6, 1.1	3
PW3	River water	93	0
PW4	River water	31	1
PW5	Soil	ND*	0
PW6	Stream water	31	3
PW7	Soil	132, 51, 45, 11, 7, 6.2, 3.6, 2.5	0
PW8	Rotten onion	ND*	1
PW9	Soil	> 250	0
PW10	Stream water	16, 12, 7.1	0
PW11	Soil	ND*	3
PW12	Reservoir water	ND*	0
PW13	Rhizosphere	ND*	1
PW14	River water	ND*	1
PW15	Rhizosphere	ND*	0
PW16	Stream water	ND*	0
PW17	Reservoir water	ND*	3
PW18	Rhizosphere	ND*	0
PW19	Rhizosphere	ND*	2
PW20	Stream water	ND*	0
PW21	Reservoir water	ND*	0

* ND, none detected.

differed in profile. This evidence strongly indicates high genetic variability amongst environmental populations of *B. cepacia*. More detailed genetic and phenotypic investigation,

such as the use of fatty acid methyl ester analysis (FAME), would allow the genomovar status of the isolates obtained from the environment in this study to be determined.

Table 2 Antibiotic resistance profiles and MIC range of *Burkholderia cepacia* isolates

Source of isolate	Ceftazidime		Gentamicin		Piperacillin		Sulphamethoxazole	
	Resistant isolates (%)	MIC range $\mu\text{g ml}^{-1}$	Resistant isolates (%)	MIC range $\mu\text{g ml}^{-1}$	Resistant isolates (%)	MIC range $\mu\text{g ml}^{-1}$	Resistant isolates (%)	MIC range $\mu\text{g ml}^{-1}$
Environment	14	1-32	29	1-256	0	1-4	70	4-256
CF patients	50	4-32	81	32->512	80	1-256	13	4->512

Fig. 1 PCR ribotyping of *Burkholderia cepacia* strains. Composite photograph, lanes numbered left to right. Lanes 1 and 20 are size markers; lanes 2-13 are environmental isolates; lanes 14-19 are CF isolates

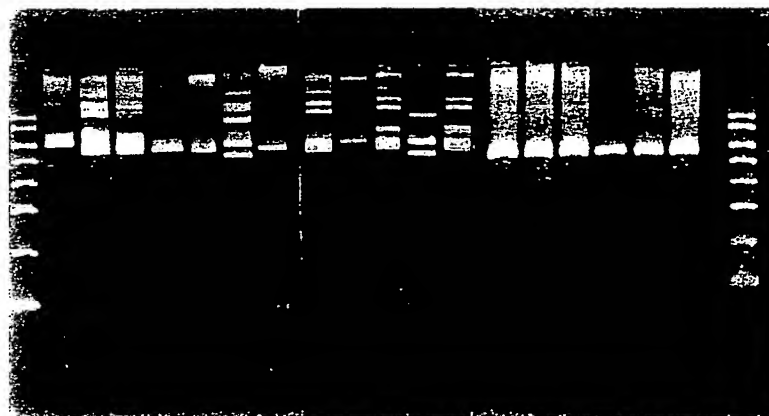


Fig. 2 Macrorestriction analysis of *Burkholderia cepacia* strains. Composite photograph, lanes numbered left to right. Lanes 18 and 19 are size markers; lanes 1-9 are CF isolates; lanes 10-17 are environmental isolates

No identity was found between the environmental *B. cepacia* isolates from South Wales and the *B. cepacia* isolates from patients attending the Cardiff CF centre, indicating that the environmental isolates differ genetically from those infecting CF patients. The environmental isolates were also found to differ from those obtained from other CF centres, from the species type strain NCPPB 2993 (ACTC 25416), and also from other environmental isolates obtained from the National Collection of Industrial and Marine Bacteria (NCIMB) and the National Collection of Plant Pathogenic Bacteria (NCPBB). The differences would suggest that environmental *B. cepacia* isolates may form a separate population to those infecting CF patients.

With the techniques used in this study, identity between all but one of the Cardiff CF isolates was shown. These isolates can be further subdivided by *Tag* I digestion of the PCR products (Ryley *et al.* 1995). Nevertheless, the identity shown in this study between these strains, and others from Scotland and Strasbourg, both by PCR ribotyping and macrorestriction analysis, would indicate that they are closely related if not identical. The presence of a common strain amongst these centres would indicate both a common initial source, and strong evidence of person-to-person transmission (Govan *et al.* 1996).

Phenotypic differences in antibiotic resistance and the ability to cause onion maceration both between CF and environmental isolates, and within the populations, were also observed (Tables 1 and 2). Levels of antibiotic resistance were generally higher amongst CF isolates, as found by Butler *et al.* (1995). However, it is not clear whether such differences are fundamentally related to the biology of the strains themselves, or are due to the development of acquired resistance amongst the CF isolates which will have received considerable exposure to such drugs. Maceration of onion tissue was more common and generally more severe by environmental isolates, but was not exclusively caused by these isolates.

Plasmids were found in 55% of *B. cepacia* isolates, though they were more commonly found amongst the CF isolates (76% of isolates) than in environmental isolates (33% of isolates). Plasmids in excess of 100 kbp were harboured by both clinical and environmental isolates. Lennon and DeCicco (1991) suggested that large plasmids were associated with clinical isolates and high levels of antibiotic resistance but in this study, there was no evidence of large plasmids being more frequently harboured in CF isolates; indeed, many of the largest plasmids were found in environmental isolates. Strains which appeared identical by ribotyping and genomic fingerprinting were nevertheless found to differ in their plasmid content, e.g. the Cardiff CF isolates. The transfer, acquisition and assimilation of plasmid-borne genes is extensively documented, and the fact that closely related strains differ in their plasmid content could reflect differences in selective pressure, such as antibiotic treatment regimes,

and differences in the transferable plasmid-bearing lung microflora of other species in CF patients. It is not known if the plasmids are sufficiently stable to be an aid in strain identification; the plasmids in our collection are as yet cryptic.

Three main points emerge from this study. First, the environmental population is genetically very diverse. There is also considerable phenotypic diversity amongst these strains. Secondly, the clinical population of *B. cepacia* is genetically much more homogeneous and is distinct from the environmental population. The final point that can be made is that the variation both between and within environmental and clinical groups may be the result of *B. cepacia* being a complex formed by five genomic species, with the clinical population being predominantly formed by *B. cepacia* genomovar III, *B. vietnamensis* and *B. multivorans*, while many environmental isolates, particularly phytopathogenic isolates, fall into genomovar I (Govan *et al.* 1996; Revets *et al.* 1996; Vandamme *et al.* 1997). However, it would appear that such a grouping is not rigid, with genomovar I being described in CF patients and other clinical sources and some strains predominantly found in CF patients also occurring in soil (Vandamme *et al.* 1997). Although this study suggests that environmentally occurring *B. cepacia* forms a separate population to that infecting CF patients, it is limited to only 21 environmental isolates and 21 clinical isolates. A more extensive study, particularly including a wider range of less related CF isolates, would be needed to confirm the validity of these findings. The apparent acquisition of *B. cepacia* from the environment by CF patients (Cazzola *et al.* 1996), the close genetic relationship between the species type strain and the ET12 CF epidemic strain (Johnson *et al.* 1994), the ability of some CF strains to cause onion maceration, and the overlap of genomic species between the environment and CF patients (Vandamme *et al.* 1997), suggest that a greater understanding of relationships between clinical and environmental *B. cepacia* is needed. The occurrence of genomic species infecting CF patients in the environment and the ability of primarily environmental genomovars to infect man needs further investigation. The use of molecular typing techniques such as PCR and PFGE, together with animal models such as the *cfrml*HGU mouse which develops lung disease when challenged with *B. cepacia* (Davidson *et al.* 1995), may allow both a greater understanding of the relationship between clinical and environmental isolates of *B. cepacia* and their potential pathogenicity. Such information may answer the question of whether environmentally occurring *B. cepacia* pose a risk of infection to CF patients.

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